HERPES SIMPLEX VIRUS TYPE 2 LATENCY:

EXPERIMENTAL STUDIES WITH WILD TYPE VIRUS AND 13 TEMPERATURE SENSITIVE MUTANTS

BY

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SUMMARY

The aim of the work described in this thesis was to identify genetic loci of herpes simplex virus type 2 (HSV-2) with functions needed to initiate, maintain or reactivate virus from the latent infection. The experimental system adopted involved mouse footpad inoculation and 3 months later explantation of dorsal root ganglia and footpads, and used 13 of the temperature sensitive (ts) mutants derived from the HG52 strain of HSV-2. The phenomenon of "latency" was investigated in five different strains of mice in order to provide information about the possible involvement of host genetic factors in modulating HSV latent infection. The mouse core temperature (38.5°C) is non permissive for growth of the ts mutants and therefore synthesis of infectious virus is inhibited by virtue of the ts lesions.

The results indicate that ts mutants were recoverable at different frequencies from the latent state in dorsal root ganglia. The mutants ts 3, ts 5 and ts 9 were only recovered on one occasion each (1/80, 1/59, 1/66) respectively. This finding implies that these mutants are impaired in functions that affect latency. The precise nature of this impairment needs further detailed examination. A number of intracerebral inoculations were also carried out. No virus was recovered from the brain of mice 6 weeks after intracerebral inoculation with either ts 3, ts 5 or ts 9.

None of the 13 ts mutants reactivated from the ganglia as efficiently as the wt virus. Differences in the capacity of the various ts mutants to be recovered from the latent state support the notion that latency is under the genetic control of several viral genetic loci. Mouse strains were found to differ in the frequency with which these viruses

could be recovered from the latent state, e.g. "BALB/c", "BIOZZI" and "A" strains giving more frequent recovery than "C57BL/6-J" or "PIRBRIGHT": evidence for the involvement of host genetically determined factors in modulating latency. In addition no correlation was observed between the susceptibility of mice to acute infection and latency; this is another interesting aspect of the system which needs further investigation.

The mutant viruses as well as the wt were reproducibly recovered from the footpad tissues of latently infected mice, and differences in the recoverability of the ts mutants from the footpads were also observed: for example ts 5 (0/59) and ts 11 (0/58) have never been recovered from the footpad whereas ts 3, ts 4 and ts 10 were recovered more often from the footpad than other mutants and wt viruses. This makes ts 5 is a specially notable mutant, having been recovered from latency on one occasion only.

The following lines of evidence points toward the possibility that HSV can establish latent infection in the footpad and therefore in cells other than neurones: 1) the inability to detect virus in footpad tissue homogenised immediately after explantation; 2) the difference in the time of virus reactivation from the dorsal root ganglia and from the footpad (generally viruses take longer to reactivate from the footpad); 3) the fact that some ts mutants were recovered from the ganglia but not from the footpad, and others from the footpad but very rarely from the ganglia. However, the possibility that the virus is merely persistent in the footpad can not yet finally be excluded. Experiments are underway to elucidate this point.

HSV-2 was successfully reactivated from dissociated

dorsal root ganglia in culture; viral antigen first appeared in neurones and only subsequently did reactivated virus spread to non neuronal ganglionic cells. Recovery of virus from dissociated ganglia clearly shows that anatomical integrity is not required for reactivation and furthermore allows the system to be examined in a qualitative and quantitative way.

The recovered viruses were always found to have retained their ts genotype. The identity of recovered viruses (HSV-2) has been confirmed by comparing their restriction enzyme profiles to that of the originally inoculated viruses. Apart from one instance, no change in restriction sites was observed, however, shifts in the mobility of certain restriction enzyme fragments were noticed. In one isolate of ts 3, a HindIII site is missing between the l and m fragments.

Virus was never recovered from the footpad of the non inoculated side. Virus was not recovered from the left dorsal root ganglia except for one instance where wt virus was recovered from the left sided ganglia and from the spinal cord tissue explanted from a mouse showing paralysis 5 months after inoculation. In many cases virus could be recovered from ganglia not associated with the inoculated dermatome. These findings simply suggest the spread of the virus within the nervous tissue of inoculated mice and also indicate that latent infection can also be established within the spinal cord.

The work described here analyses the effect on latency of 13 HSV-2 ts mutants and also demonstrates the important modulating influence of the host genetic makeup. The state of the viral genome during latency has yet to be elucidated.

Further investigation of the latency capacity (both *in vivo* and *in vitro*) of deletion mutants, *ts* mutants in other genes and recombinants should allow precise identification of the regions of the viral genome involved in latent infection.

INTRODUCTION

Herpesviruses

Herpesviruses are very widespread in occurrence and have been isolated from humans and from many other species (Nahmias, 1972). Many have a very narrow host range but in other cases this may vary considerably both in nature and in the laboratory. Herpesviruses occur in both warm- and cold-blooded vertebrates and in invertebrates. In addition, herpesviruses have also been reported in fungus (Kazoma and Schonstein, 1972).

In the earliest writings, the word "herpes" has been used to describe a variety of clinical conditions. In recent years, herpesviruses have been associated with a number of diseases and some members of herpesviruses have been reported to induce neoplasia in their natural host and/or in experimental animals. In cell culture, some herpesviruses have been shown to be able to transform cells into continuous cell lines which may produce invasive tumours in appropriate experimental hosts (reviewed by Rapp and McCarthy, 1978).

The site of primary infection with herpesviruses varies and the viruses may remain latent in their host for the life time of the host; the actual cells harbouring latent virus may vary depending on the virus type.

A common characteristic among herpesviruses is that, i. the size of the virion is 120-150 nm in diameter and they possess a linear molecule of double stranded DNA genome with an average molecular weight 80-150 x 10^6 daltons, ii. their capsid is icosahedral and composed of (~162) hexagonal and pentagonal capsomere units, iii. they are ether sensitive enveloped viruses.

At least five distinct human herpesviruses exist:

Human herpesvirus 1 is Herpes simplex virus type 1 (HSV-1)

Human herpesvirus 2 is Herpes simplex virus type 2 (HSV-2)

Human herpesvirus 3 is Varicella zoster virus (VZV)

Human herpesvirus 4 is Epstein-Barr virus (EBV)

Human herpesvirus 5 is Cytomegalovirus (HCMV)

This Thesis deals mainly with herpes simplex virus, particularly HSV-2. However, whenever it is relevant, other human herpes viruses are also discussed.

Although the clinical syndromes associated with the infection by HSV type 1 and type 2 have long been known, it is only recently that the importance of studying these viruses in detail has been appreciated for the following reasons: a, Herpes simplex virus has been isolated from different pathological lesions (conjunctivitis, keratoconjunctivitis, mouth ulcers, stomatitis, urogenital tract infection and fever blisters). In addition some cases of aseptic meningitis have been attributed to herpes infection, b, Herpes simplex virus has become a major cause of venereal infections, c: the severity of lesions and syndromes they induce has become recognised as life threatening to the newborn and to immunologically compromised hosts (Rawls, 1973), d, it is thought that HSV is aetiologically linked to urogenital cancer particularly carcinoma of the cervix, (Roizman and Frenkel, 1976), e, the virus has the ability to induce latent infection and remain within the body for the life of the individual, f, up to now and despite all efforts, there is no real prospect for eradication of these viruses; however, several antiviral compounds particularly acyclovir (ACV) have been shown to possess substantial antiviral activity in limiting virus replication, but at present none seem to affect the

established latent infection (Blyth et al., 1979; Field et al., 1979) g, the development of various molecular techniques has made the manipulation of these viruses much easier.

The two types of herpes simplex virus (HSV-1 and HSV-2) are related, with great similarities in their molecular and biological properties, yet there is a significant genetic divergence and the two types can be distinguished by a variety of immunological tests including neutralization and by the differences in the molecular weight of virus induced polypeptides (Killington et al., 1977) and on the basis of the pattern of their DNA restriction endonuclease fragments (Hayward et al., 1975; Skare et al., 1975; Lonsdale, 1979). There is approximately a 50% homology of genetic relatedness between the two serotypes (Kieff et al., 1972; Ludwig et al., 1972)

Herpes Simplex Virus (Structure and Components)

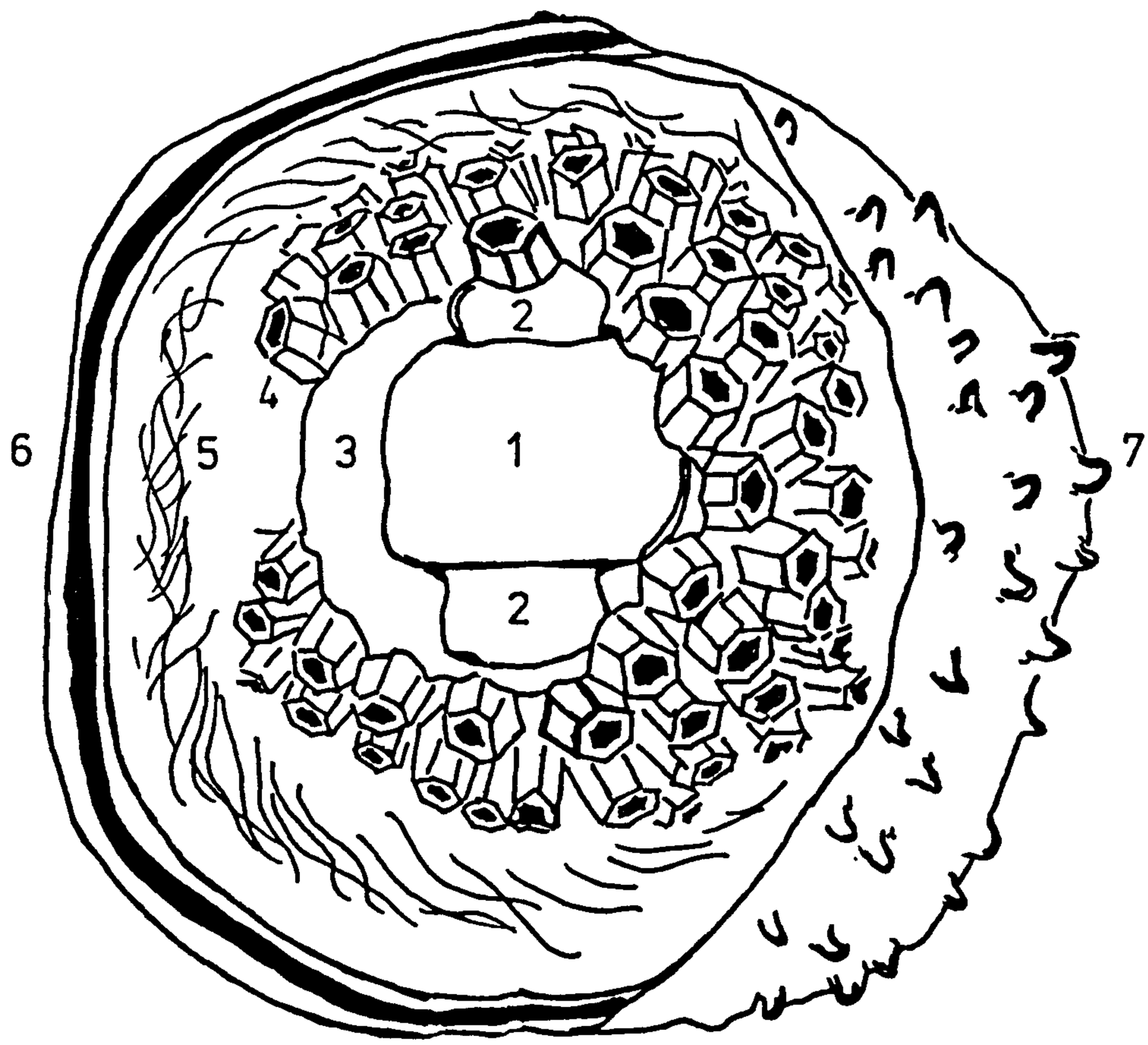
The virion

The major structural features of HSV are 1, Core; 2, Capsid; 3, Tegument and 4, Envelope. A diagrammatic illustration of the virion is shown in Figure (1). The virion diameter ranges between 120-180 nm. The virion diameter, however, is considerably affected by the diameter of the nucleocapsid and by the volume of the tegument as well as by discontinuities in the virion envelope (intact virion has smaller diameter than the disrupted particles).

In sectioned virion preparations, the core appears as electron opaque, and although the precise configuration of the DNA in the core is not known, it has been shown that the DNA occupies a toroidal compartment in the core and that a "spindle" of electron-dense material probably of

FIGURE 1

DIAGRAMMATIC ILLUSTRATION OF HERPES SIMPLEX VIRUS STRUCTURES



1. DNA
2. core
3. pericore
4. capsid
5. tegument
6. envelope
7. spikes

proteinaceous nature, extends through the whole of the toroid (Furlong et al., 1972). It has also been shown that HSV-1 capsids which contain DNA differ from those lacking cores with respect to certain virion polypeptides (VP), where VP22a appears to be on the surface of the capsid while VP21 is a possible core protein (Gibson and Roizman, 1972). The polyamine, spermine may also represent a core component packaged within the capsid (Gibson and Roizman, 1971).

The average diameter of HSV capsids is approximately 100 nm. It encloses the core and is composed of smaller units, the capsomers. The total number of the latter is 162 arranged as an icosahedral structure. Electron microscopy reveals two groups of capsomers, hexomeric and pentameric (Wildy et al., 1960). The nucleocapsid protein appears to be in the space between the capsid and the core. It is thought that the nucleocapsid protein may interact with the core and maintain its central position within the capsid (Roizman and Furlong, 1974).

The material located between the capsid and the envelope is defined as the tegument (Roizman and Furlong, 1974). In thin sections of virions or budding particles, the electron-dense tegument has no distinctive features, however, in negatively stained preparations, tegument can have a fibrous structure (Wildy et al., 1960; Morgan et al., 1968; Schwartz and Roizman, 1969). The tegument seems to be present in most virions, but the amount seen in the electron microscope can vary from virion to virion, even in the same cell. The overall quantity of tegument appears to be controlled at least in part by the virus (McCombs et al., 1971). Proteins present in de-enveloped virions but not in nucleocapsids containing DNA have been assigned to the

tegument. Among such proteins is the VP22 (36,000 MW) which is thought to be formed by cleavage of precursor tegument polypeptide VP22a (Gibson and Roizman, 1972, 1974).

The envelope is the virion outermost structure. It is composed of lipid bilayer and associated proteins. It contains spike-like projections (Fig.1) on its surface (Wildy et al., 1960). All the virion glycoproteins are thought to be restricted to the envelope and possibly the tegument (Roizman and Furlong, 1974). The polyamine spermidine is also associated with the envelope (Gibson and Roizman, 1971).

The Genome

The first direct chemical evidence indicating that HSV genome contains DNA was described by Ben-Porat and Kaplan (1962) and Russell (1962). Further studies, had shown that HSV DNA within virions is a single (double-stranded) linear molecule (Russell and Crawford, 1963, 1964; Becker et al., 1968; Frenkel and Roizman, 1971; Furlong et al., 1972; Graham et al., 1972) and that the intact HSV DNA molecule strands as well as the native duplex molecule are infectious (Sheldrick et al., 1973). The size (molecular weight) of the HSV genome has been the subject of much controversy and various methods used to determine the molecular weight have given values ranging between $51x10^6$ daltons (D) to $110x10^6$ (Russell and Crawford, 1963, 1964; Becker et al., 1968; Wilkie, 1973; Grafstrom et al., 1974; Wagner et al., 1974; Clements et al., 1976; Wilkie, 1976; Cortini and Wilkie, 1978). The general agreement, however, is that HSV genome is close to $100x10^6$ D in MW.

It is worth mentioning here that most of the above values were determined using HSV type 1, however, it has

been reported (Kieff et al., 1971) that the DNAs of HSV-1 and HSV-2 could not be differentiated with respect to size when cosedimented on neutral sucrose gradient . Moreover, Cortini and Wilkie (1978) found that the molecular weight of various regions which constitute the HSV-2 genome are very similar to the corresponding molecular weight in HSV-1 genome. The buoyant density of HSV-1 and HSV-2 DNAs were estimated to range from 1.725-1.727 g/cm^3 (HSV-1) and from 1.727-1729 g/cm^3 for HSV-2 (Goodheart et al., 1968; Kieff et al., 1971; Graham et al., 1972; Halliburton, 1972). Subsequently the base (guanine and cytosine) composition of the two subtypes were calculated and found to be 67 G+C moles % for HSV-1 and 69 G+C moles % for HSV-2. Some other methods were also used to determine the base composition of HSV-1 and HSV-2 DNAs such as nucleotide analysis (Ben-Porat and Kaplan, 1962; Graham et al., 1972), melting temperature analysis "Tm" (Kieff et al., 1971). In addition, Russell and Crawford (1963) described the use of ultraviolet absorption measurement.

Fragmentation

A significant property of HSV DNAs is their fragmentation upon denaturation with alkali (Kieff et al., 1971; Frenkel and Roizman, 1972; Wilkie, 1973; Biswal et al., 1974). Kieff and co-workers (1971) have reported that sedimentation of alkaline-denatured DNA from purified virus on alkaline sucrose density gradient consistently yielded several bands of single stranded HSV DNA ranging from fragments of $7x10^6$ D to intact strands of $48x10^6$ D in molecular weight. Similar results were reported by Frenkel and Roizman (1972) and in addition they also reported that of the seven bands sedimented on an alkaline sucrose density

gradient, only one band is an intact strand and the others are fragments with smaller sizes than the unit length. Possible explanations for this fragmentation property of HSV DNAs, are the presence of ribonucleotides (Hirsch and Vonka, 1974; Biswal et al., 1974), however, this has been largely confined to newly synthesized DNA as it seems probable that RNA stretches which arise in the replication are removed only gradually during maturation (Hirsch and Vonka, 1974). The other explanation is the presence of single-strand nicks or gaps (Wadsworth et al., 1976; Hyman et al., 1976; Ecker and Hyman, 1981). The role of these gaps or nicks within the DNA is unknown although some of the ribonucleotides may serve as primers in DNA synthesis. Their position in the DNA is subject to controversy and it has been concluded that the fragments are non random in size and originate at unique positions from single breaks of intact strands (Frenkel and Roizman, 1972). On the other hand, it has been proposed on the basis of the heterogenous sedimentation and electrophoretic pattern observed that the single-strand interruptions are randomly located on the genome (Wilkie, 1973; Wilkie et al., 1974; Hyman et al., 1977; Ecker and Hyman, 1981). Moreover, the intact strands were not unique (Wilkie, 1973) and in vitro pretreatment of the DNA with repair enzymes (DNA polymerase and DNA ligase) would prevent the alkali fragmentation (Hyman et al., 1977).

Arrangement of the Genome

Electron microscope studies have shown that most of the DNA molecules have one or two distinct ends indicating the linear nature of the herpes simplex DNA genome (Becker et al., 1968). Sheldrick and Berthelot (1974) described three structurally distinct molecules of HSV genome, namely,

a linear form "L", and two circular forms C_1 and C_2 (Fig.2a). The number of the L forms in a given preparation rarely exceeded 10-20% and more than 50% of the -L- molecules had a highly folded region or "bush" at one end. The length of these structural features (bushes) was estimated to account for 20% of the total length of the -L- molecules.

The C_1 molecules comprise more than 70% of the total. It is a single-stranded circle with a bush, and as in the L-form, the bush represents approximately 20% of the total contour length, however, all C_1 molecules possess a bush. The C_2 molecules are present as less than 10% of the total number of the molecules and consist of two single stranded circles, one short (SC) and one long (LC) in circumference, joined by an extensive and highly homologous duplex region (D). It has been shown that it is possible to convert C_1 molecule to L-form by heating the DNA in 50% formamide to a temperature above 70°C (the temperature at which complete strand separation of native DNA occurs), however, heating the DNA to and below 70°C will lead to the conversion of the C_1 molecule to the C_2 form. This thermal conversion of C_1 to C_2, probably involves a rearrangement of the bush in C_1 to the "stalk" (SC+D) in C_2. Since C_2 molecules arose from linear precursors, these must be present somewhere in the DNA strand - two inverted repetitions one to generate SC and the second to generate LC (Sheldrick and Berthelot, 1974). The finding that the DNA strand ends were located near the middle of the duplex region D, had suggested that the two inverted repeats were adjacent to each other and of equal length with one half of each repeat located at the interior and the other half at the end of the intact strand. The

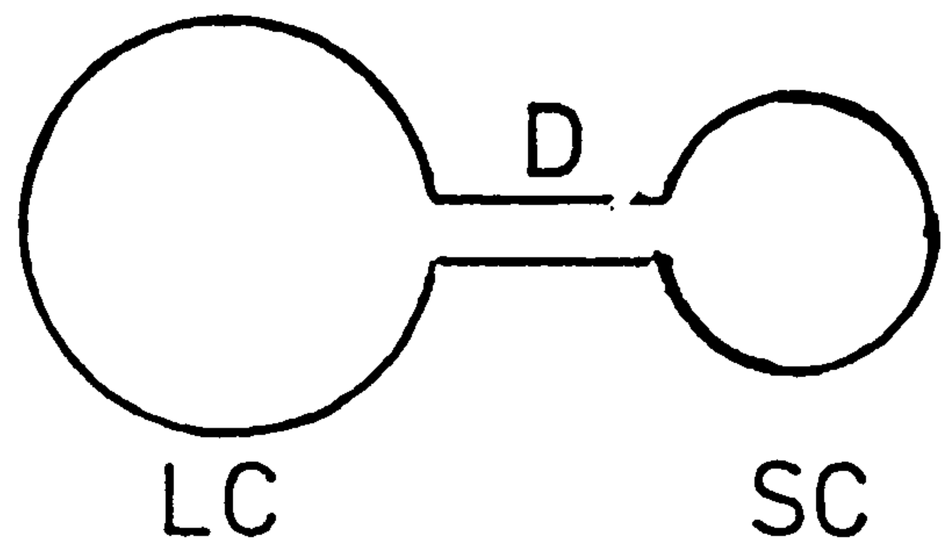
FIGURE 2

SCHEMATIC ILLUSTRATION OF:

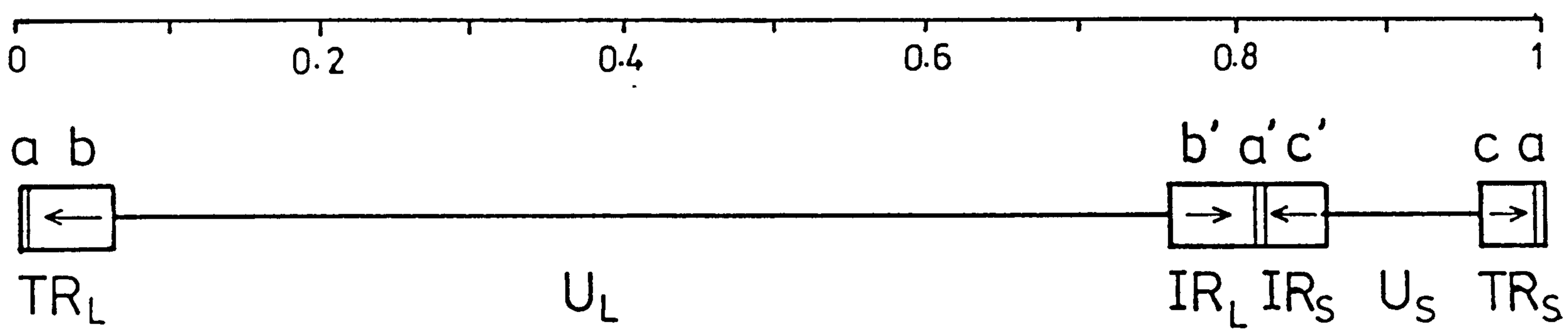
a) C_2 molecule

b) HSV genome arrangement

a



b



duplex region D, had no obvious topological features under the electron microscope (spreading condition of 50% formamide at 20°C) and contained mainly non-reiterated base sequences.

It has been observed that native HSV-1 DNA formed duplex circles at low frequency after digestion with λ exonucleases or exonuclease III, which initiate sequential digestion at the 5' and 3' termini respectively of duplex DNA (Graftstrom et al., 1974, 1975; Sheldrick and Berthelot, 1974; Wadsworth et al., 1976; Kindler and Hyman, 1979). This indicates the presence of terminally redundant base sequences. Therefore, the HSV-1 genome (Fig.2b) consists of a short and a long unique sequence (U_S and U_L) flanked by terminal and internal inverted repeat sequences (TR_S/IR_S) and (TR_L/IR_L) respectively. The sequence which is directly repeated at the genome termini (and inversely repeated at the joint) is named as the "a" sequence, while the remaining sequences within the TR_L/IR_L and TR_S/IR_S are known as the "b" and "c" sequences respectively. Other studies on self-annealed intact single strands and on partially denatured molecules of herpes simplex virus 1 DNA, confirmed the above arrangement of HSV genome (Wadsworth et al., 1975).

Isomeric Arrangement of HSV DNA

It has been proposed (Sheldrick and Berthelot, 1974) that since the terminal regions are inverted internally, an intramolecular recombination between the ends of the molecule and their repeat might readily occur. The result of such recombination will be the inversion of the entire unique regions (L or S) relative to each other. A high frequency of occurrence for such recombination events,

distributed randomly between the two sets of repeats will generate equal amounts of the four genome arrangements which result from possible combinations of inversions of the two unique regions (Hayward et al., 1975; Delius and Clements, 1976). The four possible genomic arrangements (Fig.3a) are designated P (prototype), IS and IL (inversion of S component and L component respectively) and ISL, inversion of both S and L components; (Roizman et al., 1978; Hayward et al., 1975b). The genome structure and isomerisation patterns were found to be similar in HSV-1 and HSV-2 DNA (Clements et al., 1976; Cortini and Wilkie, 1978).

Variability in HSV DNA

Nucleic acid hybridization studies and heteroduplex thermal stability analysis had demonstrated that HSV-1 and HSV-2 share 47-50% of their sequence (Kieff et al., 1972; Sugino and Kingsbury, 1976). In addition, analysis of intertypic recombinants showed that most of the recombinants can multiply and the genes of HSV-1 and HSV-2, essential for replication of the virus, are grossly colinear (Morse et al., 1978; Marsden et al., 1978). However, restriction endonuclease digests of HSV-1 and HSV-2 DNA revealed that the two serotypes differ in most of their cleavage sites (Hayward et al., 1975; Skare et al., 1975; Clements et al., 1976; Wilkie, 1976; Skare and Summers, 1971; Wilkie et al., 1977; Cortini and Wilkie, 1978; Locker and Frenkel, 1979). Moreover, intratypic strain variations in the restriction endonuclease digest patterns of both HSV-1 and HSV-2 DNA have also been observed (Hayward et al., 1975; Skare et al., 1975; Buchman et al., 1978; Lonsdale, 1979; Lonsdale et al., 1980). The major differences seen were in the form of i, absence of restriction sites leading to the fusion of two

FIGURE 3a

THE FOUR GENOME ISOMERS OF HSV-1

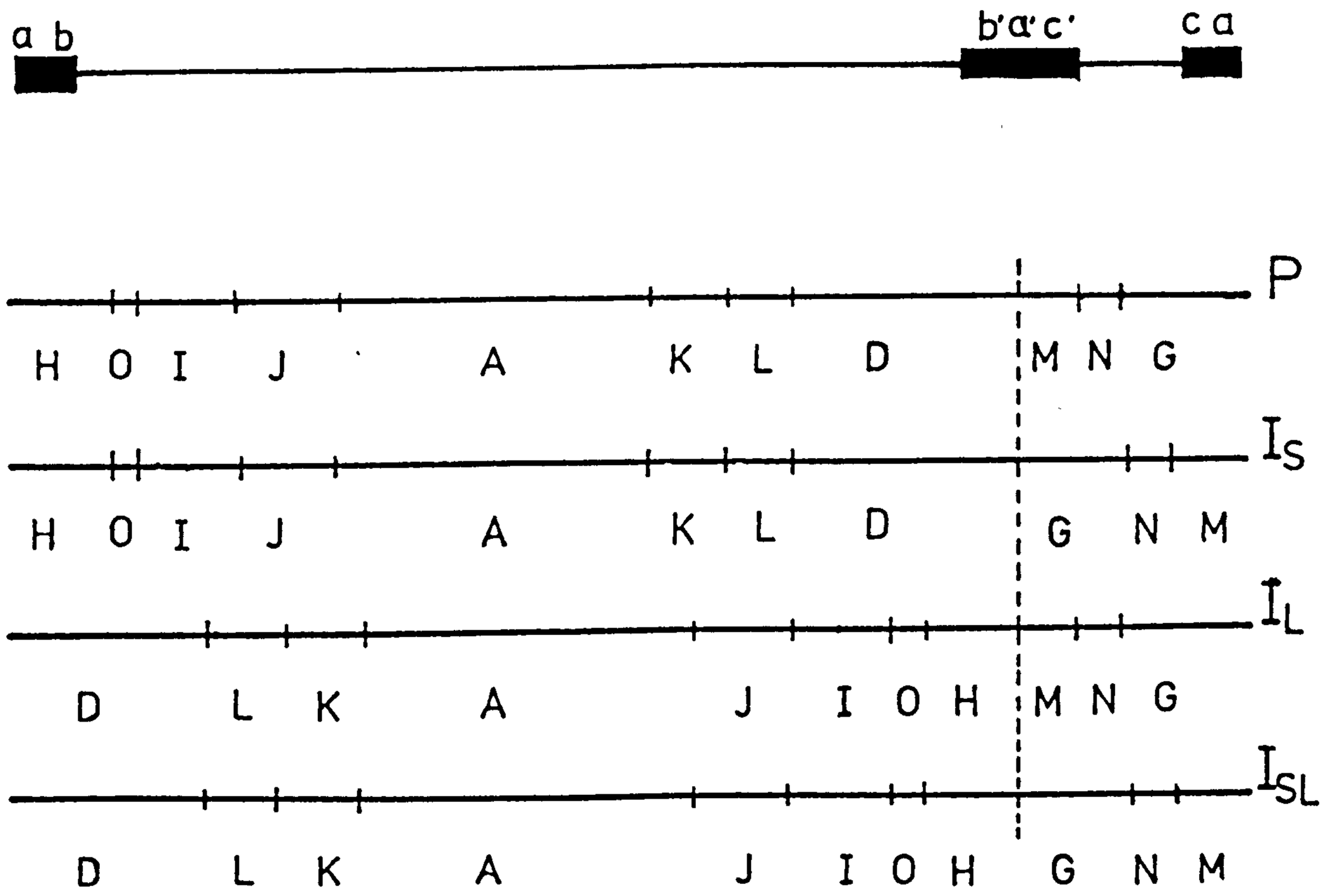
Cleavage of the prototype molecule (P) with endonuclease enzyme Hsu-1 (which cleaves DNA outside the inverted repeats) yields three fragments of interest: two terminal fragments (one on either side) H & G and one junction fragment DM. When S is inverted, that portion of the junction fragment (DM) which belongs to S component now becomes a new terminal fragment of the I_S arrangement -M-, whereas the terminal fragment of the P molecule (G) is now fused with the right end of the L component forming a new junction fragment DG. Hence, when all four genome isomers are cleaved four distinct terminal fragments are present (G, M, D, H) each of 0.5 molar ratio and four distinct junction fragments (DM, DG, HM, HG) each of 0.25 molar ratio.

P	prototype isomeric arrangement
I_S	inversion of S component
I_L	inversion of L component
I_{SL}	inversion of both S and L components

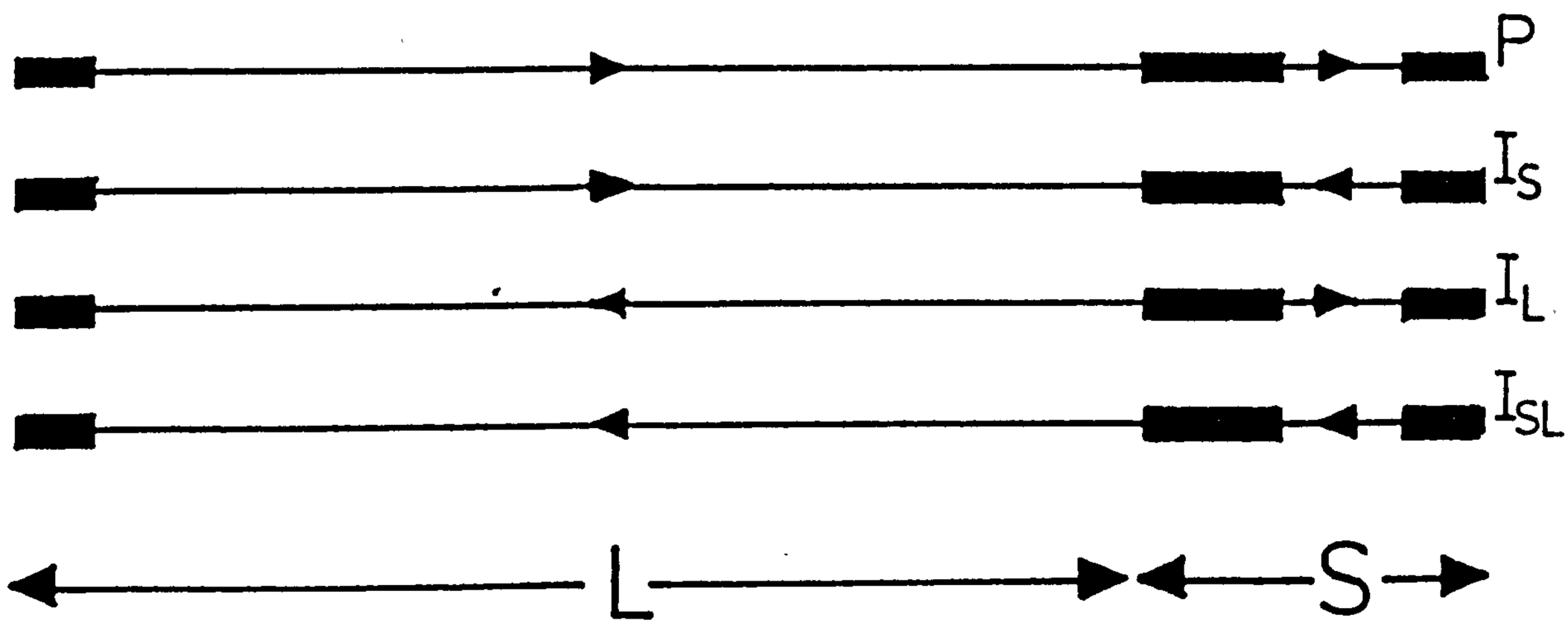
FIGURE 3b

SCHEMATIC ILLUSTRATION OF THE GENOME ISOMERS

a


a b
b' a' c'
c a
P
H O I J A K L D M N G
I_S
H O I J A K L D G N M
I_L
D L K A J I O H M N G
I_{SL}
D L K A J I O H G N M

b


P
I_S
I_L
I_{SL}
L
S

segments or gain of a new site leading to loss of a fragment compensated by gain of two smaller fragments, ii, gain or loss of small portions of fragments. This variability is reflected by intertypic variations in structural polypeptides (Heine et al., 1974; Cassie et al., 1975; Pereira et al., 1976).

Virion Polypeptides

Studies of the virion polypeptides have been published primarily for HSV-1, while similar investigations of HSV-2 polypeptides have not been published in detail. However, the number of polypeptides found in purified preparations of both viruses were approximately the same (Cassai et al., 1975). Based on the data reported by Spear and Roizman (1972) and by others (Heine et al., 1974, Powell and Watson, 1975; Cassai et al., 1975; Strand and Aurelian, 1976) HSV virions contain from approximately 15-33 structural polypeptides, which can be divided into three groups: the first group has at least six polypeptides present in nucleocapsids prior to envelopment. The second group comprises the glycosylated polypeptides most or all of which are present in the envelope of the virion. The third group contains the remainder of polypeptides and most of which are probably constitutents of the tegument.

Gibson and Roizman (1972) described three types of nucleocapsids; Nucleocapsid A and B can be isolated from the nuclei of infected cells and nucleocapsid C can be produced by stripping virions of their envelope. Nucleocapsid A (DNA deficient or empty capsid) contains four polypeptides (VP5, VP19C, VP23 and VP24), while nucleocapsid B which contains viral DNA, has two additional polypeptides (VP21 and VP22a). It has been shown that both A and B capsids have another

major protein component with a molecular weight approximately 12,000 (Heilman et al., 1979). The C capsids were found to contain all of the B capsid protein (except Vp22a), in addition it also contains several proteins present in the virion, i.e. proteins 1-3. It has been concluded that most of the virion glycoproteins are membrane associated and are in an exposed position at the surface of the virion (Olshevsky and Becker, 1972; Roizman and Furlong, 1974). In support of the latter conclusion, is the finding that antibodies directed against glycoproteins (with molecular weights of 100,000-130,000 or against glycoprotein gD (VP17) have neutralising activity (Powell et al., 1974; Cohen et al., 1978). Very little is known about the organisation of the tegument polypeptides and it has been considered that proteins external to the capsid and not in the envelope are in the tegument, and this would include proteins 1-4, 6, 6.5, 20 and 22 (Roizman and Furlong, 1974).

Variability in the antigenicity and electrophoretic mobility of the virion polypeptides among isolates of HSV-1 and HSV-2 has been observed (Cassai et al., 1975; Pereira et al., 1976). Serological tests have proved the variability in virion glycoproteins (Killington et al., 1978).

DNA Binding Proteins

Preliminary evidence for the presence of a virus specified DNA binding protein was provided by Bayliss et al. (1975) and Kudler and Hyman (1979). It was found that non-deproteinised viral DNA isolated from HSV-1 infected cells was unable to circularise following exonuclease III treatment, and that the DNA was more sensitive to digestion by a 3' than by 5' exonuclease suggesting that the proteins are more strongly associated with one strand than the other.

Restriction endonuclease cleavage profiles of highly purified but not specifically deproteinised DNA were found to be different from the published pattern (Hyman et al., 1979). More evidence for DNA binding proteins was reported by Hayman (1980) where it has been shown that polyacrylamide gel electrophoresis of purified HSV DNA (treated with ^{125}I labelled Bolton-Hunter reagent which bound to free amine group of protein but does not affect DNA) revealed four bands of protein. Electron microscopic studies using dinitro fluorobenzene/antibody complex allowed the visualisation of the protein at four sites on HSV DNA, i.e. near the ends and within the inverted terminal repeats on the DNA (Wu et al., 1979). The exact functions of the DNA-bound proteins are unknown, however, a rolling-circle mechanism has been proposed for HSV DNA replication which may utilise such proteins in intermediate structure stabilisation (Jacob et al., 1979). Recently, Powell and colleagues (1981) purified major HSV-2 DNA binding protein (ICSP 11/12) and showed that antisera prepared against the purified binding proteins inhibits (in vitro) virus DNA synthesis whereas antisera reactive with other virus proteins do not exert such effect, suggesting the involvement of ICSP 11/12 in DNA replication. In addition, these proteins were also found to cause the denaturation of the DNA helix at 40°C. More details, about the involvement of the DNA binding protein in the mechanism of DNA synthesis have been reported by Littler et al. (1983), as it has been shown that HSV-2 ts mutants defective in DNA binding protein (mutation mapped to the area of the genome known to code for these properties) were also defective in replication of virus DNA and that induction of both DNA polymerase and

alkaline exonuclease were also affected by the ts lesion in the DNA binding protein. Therefore, it has been suggested that it is likely that inhibition of DNA synthesis is due to an effect brought about by the lesion on the stability of the DNA replication complex. However, it is not known how relevant these (in vitro defined) properties are to the in vivo course of viral infection.

Polyamines

Following detergent treatment, the polyamine spermidine is removed in parallel with the envelope constitutents, suggesting that this polyamine is associated with the envelope or the tegument, however, on the other hand such treatment (non-ionic detergent and urea) left the amount of spermine unchanged, indicating that spermine is restricted to the nucleocapsid (Gibson and Roizman, 1971). The presence of the polyamines has been confirmed by McCormick (1978). It is thought that the function of the spermine is to neutralise DNA-phosphate and may play a role in condensation of viral DNA.

Lipids

Chemical analysis (Asher et al., 1969; Ben-Porat and Kaplan, 1971) revealed the presence of lipids in HSV virions. It has been shown that the infectivity of the mature virion is destroyed by phospholipase C, suggesting that the lipids are essential for infectivity and contained in the envelope (Spring and Roizman, 1968). Very little is known about the factors that might influence the lipid composition of HSV virions.

Replication Cycle

Cell Penetration

The finding that specific infectivity of naked

nucleocapsids is much lower than that of intact virions (Spring and Roizman, 1968; Rubenstein et al., 1972) clearly indicated that the virion envelope plays a major role in entry of the virion into the host cell. This has been confirmed, as ts mutations of an envelope constitituent results in the production of enveloped particles that can not penetrate into cells (Sarmiento et al., 1979). Since virion glycoproteins are exposed on the surface of the envelope (Olshevsky and Becker, 1972; Roizman and Furlong, 1974), it is highly likely that they play a role in viral adsorption to, and/or penetration of the host cell. However, the possible participation of glycoprotein C (gC) in viral adsorption has been ruled out, as a gC deficient virion can still be infectious (Keller et al., 1970; Manservigi et al., 1977). Similarly, virions lacking gB can bind to cells - although they can not initiate infection - an indication that gB is required for penetration but not for adsorption (Sarmiento et al., 1979). It has been hypothesized that gB function is to promote fusion between the virion envelope and the cell surface membrane. This has been supported by the above observation (Sarmiento et al., 1979) and by the finding that gB plays an essential role in the promotion of HSV-induced cell fusion (Manservigi et al., 1979). Glycoprotein D (gD) has been shown to be HSV-1 type specific envelope component which is involved in the attachment of the virion to the host cell plasma envelope (Vahlne et al., 1979). The mode of viral entry into the host cells is thought to take place either by i, phagocytosis, where the attachment of the virion to the host cell surface triggers their phagocytosis, which is followed by partial disruption of the virus and release of the DNA

(Dales and Silverberg, 1969) or ii, after the adsorption of the virus to the host-cell surface, the virion envelope fuses with the cell surface membrane, releasing the nucleocapsid into the cytoplasm (Morgan et al., 1968). However, the presence of virus receptors in cell-surface membranes and the identification of a virus envelope glycoprotein, that plays an essential role in cell fusion (Manservigi et al., 1977) appears to favour the second hypothesis of viral entry to the host cell.

Following removal of the envelope and entry of the virus, the virus particles appear to be uncoated by existing cellular enzymes and the naked viral DNA to be gradually transported into the nuclei (Hochberg and Becker, 1968). It has been observed that infectious DNA of HSV forms a complex with host nuclear protein, while the newly synthesised viral DNA does not possess such a property. Moreover, since this complex can be formed in the presence of inhibitors of RNA and protein synthesis, it is possible that it may be involved in the immediate early functions of the HSV genome (Rice et al., 1976).

Viral and Cellular DNA Synthesis in Herpes Virus Infected Cells

Cellular DNA

Infection of cells with herpes simplex virus results in cessation of host cell DNA and protein synthesis, and a marked reduction in host cell RNA synthesis (Ben-Porat and Kaplan, 1973). The mechanism that switches off the synthesis of these macromolecules is unknown. However, it is probably mediated by a virus-specific protein bound to cellular DNA, in the case of pseudorabies virus infected cells (Ben-Porat and Kaplan, 1965). Similarly, in HSV-2

infected cells a virion structural component has been suggested to switch off the synthesis of cellular DNA (Fenwick and Walker, 1978). Halliburton and Timbury (1976) have reported that two of the HSV-2 ts mutants (ts 9 and ts 11) were not able to switch off host DNA synthesis at the non-permissive temperature (38°C). It appears likely that these mutants are deficient in synthesis of, or lacking of, this virus specific protein(s). In a recent report, Notarianni and Preston (1982) provided information that infection of chick embryo fibrolasts with HSV-1 ts K mutant stimulates the specific set of cellular genes which encode "stress" or "heat shock" proteins. The function of the stress proteins is unknown, though it has been shown that the protein is being induced in viral infected cells where there is an overproduction of viral IE polypeptides.

Viral DNA

Viral DNA synthesis takes place in the nucleus. Evidence for that was provided by Munk and Sauer (1964); Kaplan and Ben-Porat (1964); Roizman (1969), and it has been shown that in cells fractionated immediately after a 5 minute pulse labelling with ^{3}H-thymidine, labelled DNA was found in the nuclear fraction. In Hep-2 cells infected with HSV-1 at 31°C, viral DNA synthesis started 3 hours post-infection (PI), peaking at 4-6 hours and was virtually over by 12 hours PI (Roizman, 1969). While in HSV-2 infected BHK21 cells at 37°C, viral DNA synthesis was initiated around 2 hours (PI) and continued at a rapidly increasing rate for a further 8 hours (Munk and Sauer, 1964; Wilkie, 1973). Only a small fraction of parental viral DNA (HSV-1) entered the replicative pool (Jacob and Roizman, 1977), this might be because most of parental molecules are

not uncoated (Leinbach and Summers, 1980). It appears that HSV-2 DNA synthesis in BHK cells (at 31°C) is somewhat slower, starting at 3-5 hours PI, reaching a peak at 9-11 hours post-infection (Rixon, 1977).

RNA Synthesis in Herpes Virus Infected Cells

Cellular RNA

As previously noted, infection of cells with herpes simplex virus leads to an overall decline in cellular RNA synthesis (Roizman et al., 1965; Hay et al., 1966; Ben-Porat and Kaplan, 1973). By cell fractionation and labelling at longer intervals, Wagner and Roizman (1969) were able to demonstrate that nuclear precursor rRNA (45S) synthesis decreases and its processing to 28S and 18S does not take place. Moreover, a reduction in the rate of synthesis of cellular messenger RNA (mRNA) and of transfer RNA (tRNA) in herpes virus infected cells, has also been reported (Bell et al., 1971; Rakusanova et al., 1972).

Viral RNA

The existence of virus specified ribonucleic acid in HSV infected cells has been established (Hay et al., 1966; Flanagan, 1967). It has been found that viral RNA is made in the nucleus and has a sedimentation coefficient of 50S or more and therefore, can be distinguished from the 45S cellular precursor rRNA (Wagner and Roizman, 1969). Viral RNA smaller (16S-20S) than that found in the nucleus (50S) has been detected in the cytoplasm associated with polysomes. It is suggested that this small RNA is generated by the cleavage of large precursor RNA molecules (Wagner and Roizman, 1969a). Herpes simplex virus mRNA has been found to possess 3'-polyadenylated (poly A) tails (Bachenheimer and Roizman, 1972) and the 5' termini are modified, being

capped with a methylated guanine residue joined to the RNA through 5'-5' pyrophosphate linkage. Moreover, studies on the methylation pattern of viral mRNA revealed the existence of 5'-terminal and internal, methylated nucleotides (Bartkoski and Roizman, 1976). It seems that the 5' capped ends of viral mRNA are essential for efficient in vitro translation (Jacquemont and Huppert, 1977), as it has been found that inhibition of viral RNA methylation particularly of the 5'-cap, by 5' S-isobutyl-adenosine (a structural analogue of 5-adenosyl homocystein) prevents the replication of HSV-1 by inhibiting protein synthesis.

It has been suggested that host cell RNA polymerase activity is responsible for HSV-1 immediate early RNA synthesis in the infected cell (Alwine et al., 1974). This enzyme activity has not been found as a virion component implying that initial transcription utilises a pre-existing cell coded RNA polymerase. The fact that purified herpes virus DNA is infectious (Graham et al., 1973) supports the above observation. Studies using α-amanitin (an inhibitor of cellular RNA polymerase II activity), revealed that viral transcription is blocked at all stages in infection and suggesting that this particular host enzyme is required for the replication of HSV (Alwine et al., 1974; Preston and Newtown, 1976; Costanz et al., 1977). Furthermore, it has been shown that HSV replication can occur in α-amanitin-resistant cells lines but not in α-amanitin-sensitive cell lines, in the presence of the inhibitors (Ben-Porat and Becker, 1977).

Regulation of Viral Transcription

Using inhibitors of DNA and protein synthesis to study regulation of viral genome transcription, three classes of

viral RNA have been described. The immediate early (IE) or α; Early (E) or β and the Late or γ. The synthesis of IE RNA takes place directly after infection and before the commencement of virus protein synthesis. The synthesis of this class of RNA takes place even in the presence of the protein synthesis inhibitor cycloheximide. Early transcription takes place prior to viral DNA synthesis but after the start of protein synthesis, while late (γ) transcription is dependent on prior viral DNA synthesis (Wagner et al., 1972; Frenkel et al., 1973; Clements et al., 1977; Jones and Roizman, 1979; Holland et al., 1980; Mackem and Roizman, 1981).

Splicing of Viral mRNA

In addition to 3' polyadenylation and 5' capping and methylation of viral mRNA (Bachenheimer and Roizman, 1972; Bartkoski and Roizman, 1976), a third post-translational modification of the viral mRNA i.e. "splicing" has been detected. Three instances of splicing in HSV mRNA have been detected. These are "IE" RNAs 4 (which maps across the U_S/IR_S junction); Immediate early RNAs 5 (which maps across the U_S/TR_S junction) and the major late 2.7 kb mRNA (which maps on the HindIII 1 restriction fragment in U_L region) (Frink et al., 1981; Watson et al., 1981; Rixon and Clements, 1982). The roles of the spliced RNA segments are subject to speculation and they could be involved, as in adenovirus infected cells, in the selection of certain viral RNA sequences for transport to cell cytoplasm or could be responsible for the preferential translation of viral mRNA during the late stage of infection (Berget et al., 1977).

Protein Synthesis in Herpesvirus Infected Cells

The effect of infection of cells with herpesviruses or

the overall rate of protein synthesis varies with the different virus-cell systems (i.e. infecting virus, multiplicity of infection, type of cells and their physiological state). The rate of protein synthesis in HSV infected cells undergoes an initial reduction, then an increase between 3-6 hours post-infection, and then followed by an irreversable decline (Roizman et al., 1965). These changes in the kinetics of synthesis correspond to quantitative changes in cytoplasmic polyribosomes (Sydiskis and Roizman, 1967, 1968; Silverstein and Engelhardt, 1979).

Cellular Proteins

Four criteria have been used to demonstrate the synthesis (inhibition) of cell-specific protein in cells infected with herpesviruses. First is the immune precipitation where isotopically labelled protein (cellular or viral) is precipitated by specific immunoglobulin (γ-globulin). The result of such experiments indicated an increase in the amount of radioactivity in protein precipitated by viral specific γ-globulin and a decrease in radioactivity of protein precipitated by cell specific γ-globulin (Hamada and Kaplan, 1965). Second is the disaggregation of cellular polysomes (170S) after infection and their reconstitution as (220S-270S) viral induced polysomes (Sydiskis and Roizman, 1967). Third is the ratio of various amino acids to each other in viral and cellular proteins. Uninfected cells have much higher ratios of lysine to leucine than those of the infected cells (Kaplan et al., 1970). Fourth, electrophoretic profiles of viral and cellular protein. The electrophoretic profiles in SDS-PAGE of protein synthesized by HSV-infected cells differ greatly from those synthesized by control uninfected cells

(Spear and Roizman, 1968). Host protein synthesis is rapidly suppressed after infection with herpes simplex virus (Powell and Courtney, 1975). It has been suggested that this suppression is mediated by both a virion component and a virus encoded function which is expressed early in infection (Sydiskis and Roizman, 1967; Marsden et al., 1976; Fenwick and Walker, 1978; Fenwick and Clark, 1981).

Viral Proteins

HSV infected cells synthesise a relatively large number of viral proteins in amounts sufficient to give distinct peaks in polyacrylamide gels. The number of HSV induced polypeptide species identified by using one dimensional polyacrylamide gel electrophoresis (SDS-PAGE) is about 50 (Honess and Roizman, 1973; Marsden et al., 1976; Bookout and Levy, 1980). However, a much larger number of viral polypeptides (230) has been detected by two-dimensional gel electrophoresis (Haarr and Marsden, 1981) and some of these polypeptide species are related by post-translational modification events.

The synthesis of HSV proteins takes place in the cytoplasm and many of those made late in infection migrate to the nucleus (Spear and Roizman, 1968) where they assemble into nucleocapsids and then into mature virions. The factor(s) responsible for the migration of viral proteins to the nucleus are not clearly understood. Based on the time of maximum synthetic rate and dependence on prior viral protein or DNA synthesis, HSV specific polypeptides have been classed into 3 groups, corresponding to the three transcriptional phases, i.e. α (IE), β (E) and γ (L); The α or immediate early polypeptide group are those synthesised immediately after removal of the cycloheximide (that has

been present from the start of infection); Their highest rate of synthesis is between 3 and 4 hours PI and their synthesis requires no prior infected cell protein synthesis (Honess and Roizman, 1974; Powell and Courtney, 1975). The α-polypeptide group consists largely of non-structural species.

Synthesis of "Early" or β polypeptides requires the presence of functional α polypeptides, in addition to new RNA synthesis (Honess and Roizman, 1974). Their rate of synthesis peaking between 5 hours and 7 hours PI; Functional β-polypeptides shut-off residual host synthesis as well as the synthesis of immediate early proteins. They are also required for regulating the synthesis of new viral RNA coding for the next groups the "Late" or γ polypeptides. The β group of polypeptides consist of both minor structural and non-structural species. Viral DNA polymerase (Honess and Roizman, 1975; Powell and Purifoy, 1977), thymidine kinase (Garfinkle and McAuslan, 1974; Leiden et al., 1976) are β polypeptides.

The late or γ group consist of major structural polypeptides. However, a number of the polypeptides in this group appear to be non-structural (Morse et al., 1978). Synthesis of γ polypeptides occurs at increasing rate until 12 hours PI and also requires the presence of functional polypeptides belonging to α and β groups (Honess and Roizman, 1974).

It has been concluded (Marsden et al., 1976) that the regulation of protein synthesis in HSV infected cells, is more complicated than the scheme of cascade regulation (α, β, γ) proposed by Honess and Roizman (1974). In support of the early conclusion is the identification of 9 classes

of virus-induced polypeptides which were dependent upon the DNA phenotype of the ts mutants (Marsden et al., 1976). Moreover, 2 classes of β polypeptides have been identified in HSV-2 infected cells (Pereira et al., 1977), while HSV-1 induced polypeptides have been classified into six distinct classes of which one can be subdivided on the basis of kinetics and sensitivity to arabinosyladenine (a DNA synthesis inhibitor) which can exhibit a variety of enzymes (Pedersen et al., 1981).

The polypeptide species synthesised in HSV-1 and HSV-2 infected cells, show an extensive correspondence to each other. However, there are slight differences in the apparent molecular weight and hence in their mobility in the polyacrylamide gels (Killington et al., 1977). Through the analysis of the viral polypeptides and of the restriction endonuclease fragment profiles of HSV-1 x HSV-2 intertypic recombinants, the map locations of individual polypeptides have been determined (Marsden et al., 1978; Morse et al., 1978; Preston et al., 1978; Marsden et al., 1982). The results of such analysis reveals that genes coding for all classes of polypeptides map in both the L and S regions of the genome. The map positions of the polypeptides is well correlated with the map position of corresponding mRNAs. Moreover, the sizes of in vitro translation products correlate with the sizes of corresponding in vivo products (Easton and Clements, 1980).

Modification in Herpesvirus Induced Proteins

The mobilities (in polyacrylamide gel electrophoresis) of the in vitro translated HSV-1 and HSV-2 polypeptides differ slightly from their putative polypeptides translated in vivo (Preston, 1977), suggesting a post-translational

modification of viral proteins. At least three kinds of post-translational modification may alter the mobility of virus polypeptides: i, rapid post-translational cleavage, ii, slow post-translational cleavage (determined by intracellular translocation or occurring during assembly of the virion), iii, conjugation or addition of prosthetic groups (e.g. glycosylation, phosphorylation, acetylation, sulphation, methylation). Such modification may be required for normal function of enzymes or other non-structural proteins. Preliminary evidence (Honess and Roizman, 1973) had indicated that HSV-1 (F) infected cell polypeptides do not undergo rapid post-translational cleavage similar to that seen in poliovirus infected cells, while slow post-translational changes in the mobility of at least two polypeptides were noticed. However, rapid post-translational processing of some of the HSV-1 and HSV-2 specified polypeptides has been observed (Morse et al., 1978). Moreover, experiments involving the use of chymotrypsin inhibitor tosyl-phenylalanyl-chloromethyl ketone (TPCK) revealed that at least 14 HSV-1 polypeptides are subjected to rapid post-translational modification (H.S. Marsden, personal communication).

Phosphorylation of viral specified polypeptides has also been reported (Marsden et al., 1978; Bookout and Levy, 1980) and of the 22 phosphorylated HSV-1 polypeptides, nine were found to be major species. It is possible that phosphorylation may mediate activity of regulatory proteins in HSV infected cells, since certain of these proteins, including Vmv 175, are phosphorylated. Wilcox et al. (1980) have demonstrated that the affinity of proteins for DNA is affected by their phosphorylation.

Glycosylated HSV-1 proteins are found in the virion envelope and also in the plasma membrane (Marsden et al., 1976; Spear, 1976). Four major antigenically distinct glycoproteins have been identified, namely gA/gB, gC, gD and gE (Spear, 1976; Roizman et al., 1977; Baucke and Spear, 1979).

Glycoprotein A and gB share antigenic determinants and it has been proposed (Eberle and Courtney, 1980) that gA is an intermediate in the gB glycosylation. There is evidence that all glycoproteins mentioned above are sulphated with the glycoprotein E being the major sulphated species (Hope et al., 1982). Recently, a number of minor glycoproteins and a new major glycoprotein (designated gY) have been found in HFL cells infected with HSV-1 (Palfreyman et al., 1982). At present six types of HSV-2 glycoproteins have been described (Marsden et al., 1978; Ruyechan et al., 1979) gA/gB, gC, gD, gE, gF and 92K glycoprotein. However, it is not clear whether the gC glycoprotein which had been mapped in U_L region, is in fact the 92K protein described by Marsden et al. (1983).

DNA Replication

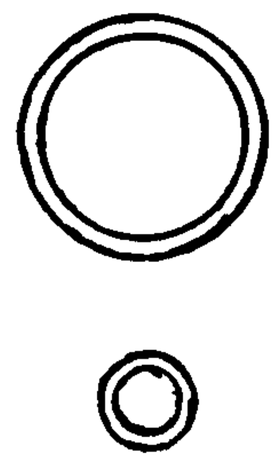
Different molecular structures of HSV-1 DNA have been isolated from infected cell nuclei. Most of these forms were indicative of replicative intermediates. The topological features of these structures as seen under electron microscope are presented in Fig. 4 after Jacob and Roizman (1977). Some of the above molecules, namely the rapidly sedimenting molecules which may correspond to those large tangled masses and molecules with single stranded regions, have been confirmed to be precursors of mature unit length of HSV-DNA (Hirsch et al., 1976; Shlomai et al.,

FIGURE 4

CONFORMATION OF HSV DNA DURING REPLICATION

CIRCLES: full size

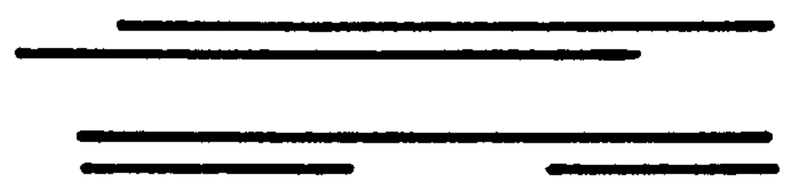
s component size



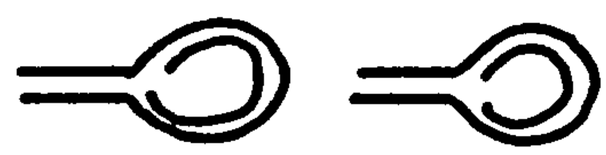
SINGLE STRANDED REGIONS:

terminal

internal

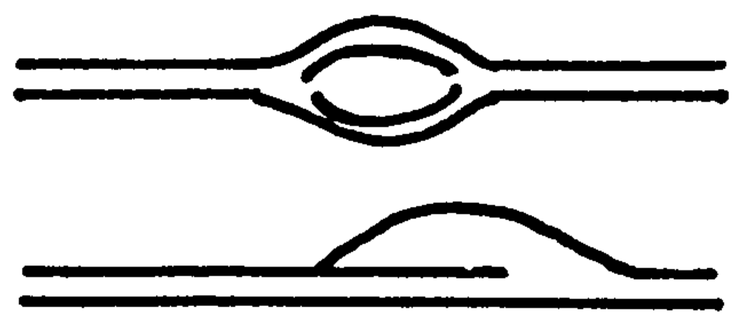


LARIATS

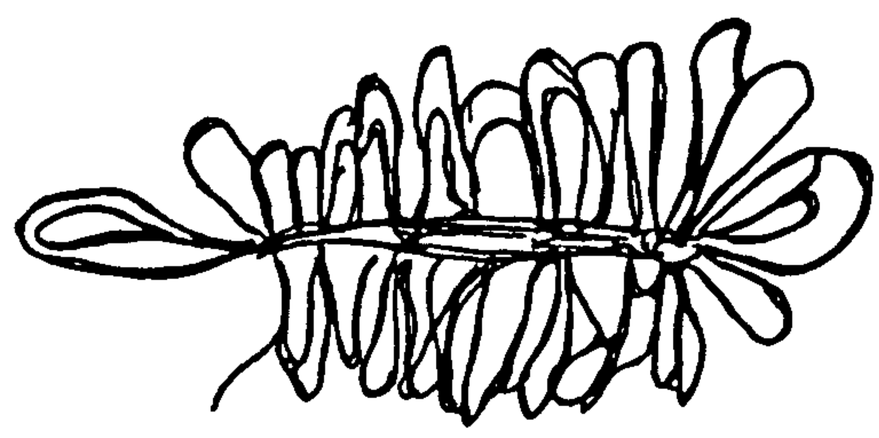


LOOPS: eye loops

D loops



LARGE REPLICATING FORMS



1976; Jacob and Roizman, 1977).

Restriction endonuclease analysis of DNA concatemers generated during HSV DNA replication (Jacob et al., 1979) show that the concatemeric DNA molecules are aligned in "head-to-tail" orientation. On the basis of the above observations, a rolling circle mechanism for the replication of HSV DNA (i.e. continuous synthesis of one strand and discontinous synthesis of the other strand) have been proposed by Jacob et al. (1979). Similar "head-to-tail" concatemeric arrangements were observed in PsRV DNA (Ben-Porat and Rixon, 1979) which is now thought to have only one main origin of replication (near or at the end of the inverted repeats of the molecule) from which replication proceeds undirectionally. The observation that PsRV DNA is largely found in circular forms during the first round of replication supports the rolling circle mechanism of replication (Ben-Porat and Veach, 1980).

The origins of replication on HSV-1 DNA have been located both at terminal and internal sites (Shlomai et al., 1976; Hirsch et al., 1977; Spaete and Frenkel, 1982; Stow, 1982). It has also been shown that purified monomeric repeat units, corresponding to map units 0.94-1.00 could replicate to generate defective multimers using only a helper virus trans function (Vlazny and Frenkel, 1981). The ac sequence of HSV genome, seems to contain the recognition site for cleavage to unit length molecules generated by rolling circle mechanism (Jacob et al., 1979), and for the encapsidation of the mature product (Vlazny et al., 1982). More recently, it has been found that the signals required for the encapsidation of HSV-1 DNA reside within the 'a' sequence (Stow et al., 1983).

Virion Assembly and Release from the Cell

The mechanism of HSV maturation is not well understood but there is evidence that the assembly of herpesvirus nucleocapsids takes place in the nucleus of the infected cells, as it has been shown by EM studies that empty capsids tend to accumulate in nuclei prior to the appearance of capsids with dense cores (Watson et al., 1964; Nii et al., 1968). Experiments designed to study the assembly of viral proteins and DNA into mature virions revealed that capsid proteins, synthesized in the cytoplasm are transported to the nucleus where they are assembled into the characteristic capsid (Olshevsky et al., 1967; Spear and Roizman, 1968).

Few, biochemical studies of nucleocapsid assembly have been reported, and studies using ts mutants of HSV-1 and HSV-2 have been carried out (Schaffer et al., 1974; Cabral and Schaffer, 1976; Atkinston et al., 1978; Dargan and Subak-Sharpe, 1983). The primary site of nucleocapsid envelopment is the inner nuclear membrance (Morgan et al., 1959). However, it has been proposed that nucleocapsids of HSV-2 may acquire their envelopes by de novo deposition within the nucleus, instead of by budding through the inner nuclear membrane (Atkinson et al., 1978).

Release of herpesvirus can take place either by budding (reverse pinocytosis) through the cytoplasmic membrane (Morgan et al., 1959) or by transportation through pores which connect the endoplasmic reticulum directly with the extracellular fluid (Schwartz and Roizman, 1969a). Moreover, virus transmission from cell to cell may take place due to fusion of neighbouring cell membranes resulting in formation of synctyia (Brown et al., 1973; Timbury et al., 1974).

HSV-2 TEMPERATURE SENSITIVE MUTANTS

HSV-2 Temperature Sensitive Mutants

In this section, the temperature sensitive (ts) mutants derived from HSV-2 (wt) strain HG52 will be discussed. It must be noted that ts mutants derived from HSV-1 and from other strains (186) of HSV-2 have also been isolated and described (Schaffer et al., 1971, 1974; Brown et al., 1973; Manservigi, 1974; Esparza et al., 1974).

Using the mutagen, 5-bromodeoxyuridine (BUdR) at a concentration of 5 ug/ml, Timbury (1971) isolated 33 independent temperature sensitive mutants from the HG52 strain of HSV-2; and on the basis of complementation tests (infectious center assay) the mutants were assigned into 10 different complementation groups. Later another three complementation groups of ts mutants were added and described bringing the total number of complementation groups to 13 (Halliburton and Timbury, 1976). The mutants were designated as ts 1 to ts 13 and found to grow well at the permissive temperature (PT) 31°C (the temperature at which they were first isolated), but no growth was noticed at the non-permissive temperature (NPT) 38°C (Halliburton and Timbury, 1973, 1976). Limited cpe (cell rounding), however, has been observed in cells infected with the mutants (except ts 9 and ts 11) at 38°C. Further, all the mutants absorbed to cells just as well as 38°C as at 31°C.

Electron microscopic studies of cells infected with the mutants at the NPT, revealed that all the 13 ts mutants were unable to produce complete electron dense nucleocapsids. On the contrary, all other types of capsids (empty, partial, dense, enveloped) were observed in cells infected with the ts^+ (wt) virus at 38°C (Atkinson et al., 1978). On the basis of the type of capsid (if any)

produced, ts mutants were classified into three classes: I. those producing no capsid structures (ts 2, 7, 9, 10 and ts 12), II. those producing empty and partial-cored capsid (ts 1, 6 and ts 13), and III. those producing empty, partial and dense-cored capsid (ts 3, 4, 5 and ts 11). In addition, an array of microtubules - a characteristic feature of HSV-2 infection - were observed in the nucleus and to a lesser extent in the cytoplasm of cells infected with the mutants except those infected with ts 6 or ts 12. Although HSV-2 ts mutants had exhibited a variety of defects in morphogenesis, these defects could not be correlated completely with the DNA phenotype as both DNA positive and DNA negative mutants showed these defects at 38°C (Atkinson et al., 1978). However, there is generally a tendency for DNA-negative mutants to show more defects than DNA positive mutants in both morphogenesis and polypeptide synthesis. Support for this conclusion comes from unpublished observations (H.S. Marsden) which indicate that DNA negative mutants show multiple defects in synthesis of polypeptides at 38°C, while DNA positive mutants have polypeptide profiles similar to that of the wt virus, except ts 3 and ts 12 which also possess multiple defects (Table 1). However, Halliburton and Timbury (1973, 1976) have shown that DNA synthesis in ts 12 and also in ts 13 (both mutants considered DNA positive) was remarkably reduced to an extent of 90% less than the wt. In the study reported by Timbury and Calder (1976), a third marker (syncytial plaque morphology or syn) was introduced into some of the ts mutants by backcrossing the ts mutants to a syncytial but not temperature sensitive mutant of wild type virus. This was used to order the ts mutations and the results showed that ts mutants of HSV-2

TABLE 1

SYNTHESIS OF POLYPEPTIDES AT 38°C RELATIVE TO THE AMOUNT SYNTHESIZED AT 31°C (H. MARSDEN, UNPUBLISHED DATA)

POLYPEPTIDES	MUTANTS												
	9	10	12	3	6	8	2	11	1	7	13	4	5
182		⇧*	⇧*										
157		▼	▼	▼	▼	▼	▼		▼	▼			
146		▼	▼		▼								ID
138				▼		▼	ID	▼					
134/132	▼												
127				⇧								⇧	
118													
66/65		▼*	▼*	▼	▼	▼	▼	▼	ID		▼*		
50		▼	▼	▼	▼	ID	▼			ID			
42.5								▼					
41.5		▼	▼	▼	▼	▼	▼		▼				
39.5		▼	▼	▼	▼	▼	▼	▼	▼				
38/37.8		▼	▼	▼		▼	ID	▼	ID		ID		
29.5		▼	▼	▼	▼	▼	▼		▼	▼			
Viral DNA synthesis	−	−	+	+	−	−	−	−	−	−	+	+	+
No. Polypeptides affected	14	9	9	9	7	7	6	5	4	2	1	1	0

▼ Polypeptide produced in significantly reduced amounts.

□ Polypeptide produced in normal amounts.

⇧ Polypeptide synthesis stimulated. * MW reduced

■ Polypeptide not detected. ID, Insufficient data.

recombine and many do so with relatively high frequency.

It is noteworthy that the L unique region comprises about 70% of the total DNA, thus there is a strong probability that most of the mutants isolated would lie within the L region (Timbury and Calder, 1976). Experiments with intratypic and intertypic marker rescue analysis confirmed the above assumption and indicated that the physical map (Chartrand et al., 1981) correlates very well with the genetic map (Timbury and Calder, 1976) except for ts 12 which possess two separate ts mutations (Fig. 5a and b). Ts 13, in addition to ts 12, is also known to be a double mutant with one lethal mutation (at 0.64-0.70 fractional length) affecting the thermal stability of the virion and a second non-lethal ts mutation (at 0.12-0.21 fractional length) in the viral (DNase activity) exonuclease gene (Moss et al., 1979). All other ts mutants of the HG52 strain show variable activity to induce this nuclease activity (Table 2) under the restrictive conditions (Moss et al., 1979; Moss, 1982).

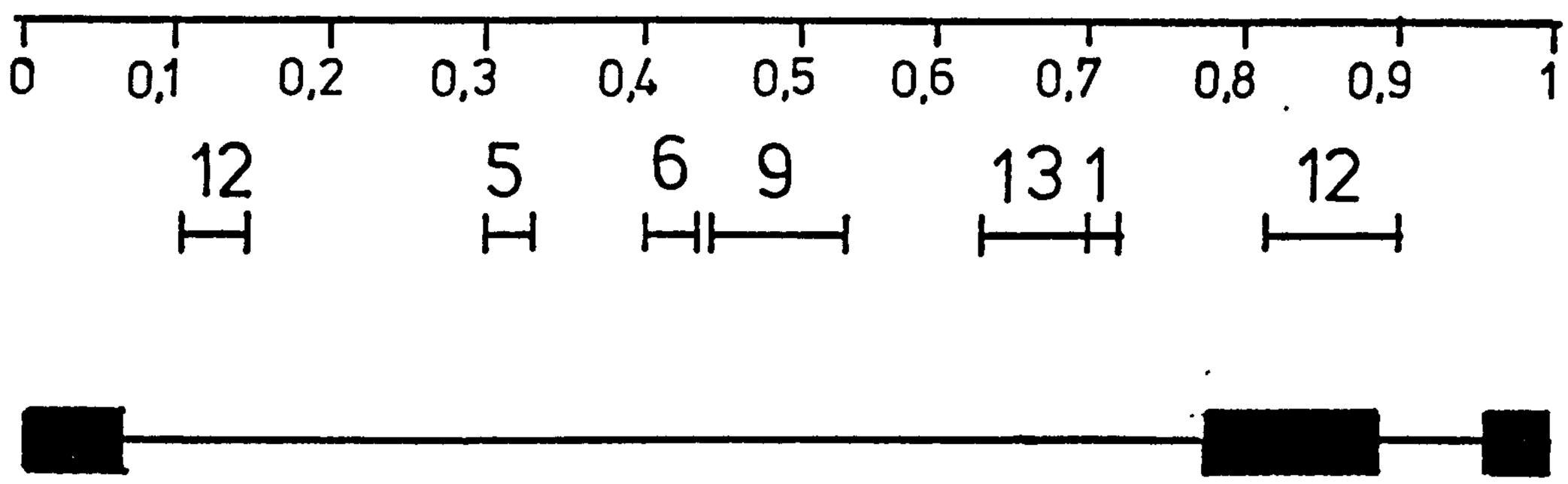
Studies with DNA polymerase activity of the mutants under low salt conditions (30 mM-KCl) and high salt conditions (100 mMkcl) at the permissive and non-permissive temperature revealed that both DNA-positive and DNA-negative mutants are able to induce DNA polymerase activity in BHK cells at 31°C; and all DNA-positive mutants do so at the NPT (38°C) (Hay et al., 1976). Moreover, while ts 6 and ts 9 (DNA-negative mutants) failed to induce this activity at the NPT, ts 11 (DNA-negative) induced only a small amount of DNA polymerase at the same temperature. On the contrary, ts 1 induced more DNA polymerase than the wt at both temperatures. The results suggested that at least three

FIGURE 5

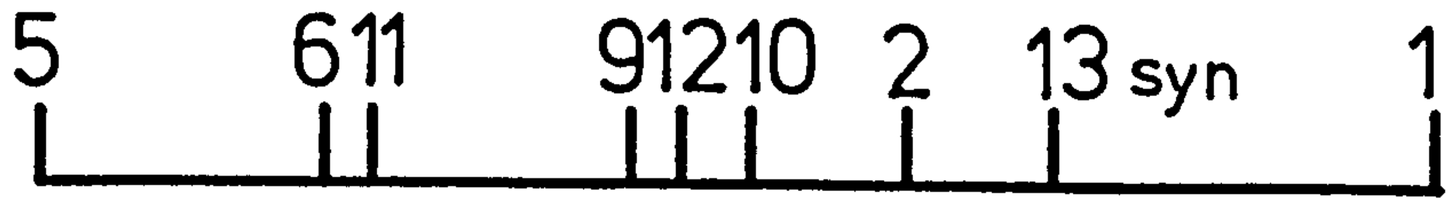
a) physical map for some of the ts mutants of HSV-2 (strain HG52)

b) genetic map

a



b



functions (ts 6, ts 9 and ts 11) were required for regulation and full expression of this enzymatic activity. In the same study, Hay et al. (1976), assay for viral pyrimidine deoxyribonucleoside kinase activity (TK) showed that mutants ts 1 to ts 10 are capable of producing this activity at both permissive and non-permissive temperatures. However, the actual amounts of the enzyme produced were not reported.

It has been reported that HSV type 2 virus, inactivated by UV radiation, had the ability to transform hamster embryo cells in vitro, and that these transformed cells were oncogenic upon inoculation into hamsters (Duff and Rapp, 1973). Utilizing the fact that in ts mutants, infectious virus synthesis is inhibited at 38°C by virtue of the ts lesion while the remaining viral gene activities remain unimpaired, Macnab (1974) successfully induced transformation of rat embryo cells with ts mutants (without UV radiation) of HSV-2 at the NPT. The result of this study revealed that ts 1 and ts 7 (DNA-negative) and ts 3, ts 4 and ts 5 (DNA-positive) are transformation positive, while all other mutants were not able to induce cell transformation (Macnab, 1974). Attempts to demonstrate infectious virus in the transformed cell line gave negative results, but HSV specific antigens were demonstrated by indirect immunofluorescence. Recently, it has been shown that HSV-1 can be rescued from a rat tumour (induced by a HSV transformed cell line) by injecting the tumour with intertypic virus HSV-2, ts 1 (Park and Macnab, 1983). Furthermore, two of the transformed cell lines, namely RE1 and RE7 (ts 1 and ts 7 transformed rat embryo cell lines respectively) and HSV-2 strain 333 proved to be tumourigenic

after injection (S.C.) into highly immunosuppressed inbred rats (Macnab, 1979). A summary of HSV-2 ts mutant characteristics are listed in Table 2.

TABLE 2

PROPERTIES OF HSV-2 (HG52) TEMPERATURE SENSITIVE MUTANTS

Virus	Log phase (hr)	Max. v. yield (hr)	Max. v. yield (hr)	CPE	Viral DNA synth.	(Switch off) Cellular DNA synth.	Effect on histone synth.	Thermostability	DNase activity (nuc)	DNA polymerase (DP)	Thymidine kinase (TK)	Transformation of rat cells	Polypeptide profile
	31°C	31°C	38°C	38°C	38°C	38°C	38°C	52°C	38°C	38°C	38°C	38°C	38°C
ts1	10-12	18-24	No growth	c	-	e	NI	Stable	nuc+	+@	+	+	Defect
ts2		18-24		c	-	ve	NI	Labile		+	+	-	Defect
ts3		18-24		c	+*	e	50% redu	Labile		+	+	+	Defect
ts4		18-24		c	+*	e	NI	Labile		+	+	+	wt
ts5		18-24		c	+*	e	50% redu	Stable		+	+	+	wt
ts6		18-24		c	-	ve	50% redu	Labile		-	+	-	Defect
ts7		18-24		c	-	e	NI	Stable		+	+	+	Defect
ts8		18-24		c	-	e	50% redu	Stable		+	+	-	Defect
ts9		18-24		-	-	f	unaffected	Stable		-	+	-	Defect
ts10		18-24		c	-	ve	50% redu	Labile		+	+	-	Defect
ts11		24		c	-	f	NI	Stable·		±	NI	-	Defect
ts12		24		c	(+)	ve	NI	Labile·		+	NI	-	Defect
ts13		24		c	(+)	ve	NI	Labile·	nuc-	+	NI	-	wt
wt	2-3	11	6	++	+	e	50% redu	Stable	nuc+	+	+	+	wt

c cytopathic effect comparable to wt was observed (but virus yield decreased by time due to heat inactivation)
* reduced DNA synthesis (10-20% less than the wt)
(+) DNA synthesis reduced (90% less than the wt)
e efficient)
ve very efficient) to switch off host cell-DNA synthesis
f fails)
NI no information
· thermostability was checked at 39°C and not at 52°C
@ enzyme induced in amount greater than the wt at both temperature (31°C and 38°C)
± enzyme produced in a very little amount

Halliburton and Timbury, 1973, 1976
Macnab, 1974
Hay et al., 1976
H.S. Marsden (unpublished data)
Mose et al., 1979
Moss, 1982

HERPES SIMPLEX VIRUS LATENCY

Terminology and Definition

The terminology of viral infection continues to be controversial, though it is generally agreed that viral infections are of two main types: I. Acute infection, as a result of virus interaction with permissive cells. Normally this type of infection is productive and leads to cell death which is reflected in the individual host as an overt infection which is usually cleared by effective immune response. II. Prolonged infection, where a degree of equilibrium is established between the virus and the infected host. Three subtypes of such infection can be distinguished. First, after infection the virus is retained within the host but in a non-detectable form, (i.e. by using present techniques) and shedding of the virus to the outside occurs sporadically, e.g. as a result of reactivation of HSV or VZV in dorsal root ganglia (DRG). This type of infection is referred to as "latent infection" and the phenomenon is known as latency. The second type of infection is where the virus persists, replicates and continues to be shed often throughout the life of an individual host in the presence of an immune response e.g. adenovirus in human tonsils. Third, a typical infection often in the presence of a defective immune response when prolonged continuous virus growth takes place, in for example Hepatitis B carrier, SSPE. Some other terms have alternatively been used for the purpose of the definition of the above types of infection (Smith and Ritchie, 1980), for example the term "inapparent infection" has been used to describe the whole area of infections which give us no overt sign of their presence. The term 'subclinical' can be used as an alternative, particularly in human medicine. According to this scheme of terminology,

latent infection is an inapparent infection, whereas "occult virus" is used to describe the case where virus particles can not be detected and in which the actual state of the virus can not as yet be ascertained. However, the simplest definition of latency is whereby an organism is infected with a virus but yet shows no apparent disease or sign of infection. The phenomenon, latency, is widespread among the herpesviruses group. Based on the behaviour of viruses in their natural hosts, Wildy and co-workers (1982) constructed a list of herpes viruses that show classical latency (Table 3). One criterion that has to be satisfied before a virus is acceptable on the list is evidence that it can remain latent in dorsal root ganglia (the anatomical site and relation to other organs of these ganglia is shown in Figure 6 and 7) and can be reactivated.

In this chapter, the available data concerning HSV latency will be reviewed, while the latent infection by other herpsviruses will be discussed later.

Latent HSV Infection in the Natural Host

Almost from the time of their discovery, herpes viruses have been known to cause latent infection, and even before the causative agent for herpetic fever blisters was identified as a virus, its ability to establish latent infection was postulated (Goodpasture, 1929). Recurrent herpes is produced by both types (HSV-1 and HSV-2) of virus. Moreover, patients may be infected with both types and have recurrences caused by either or both viruses (Wheeler, 1975). The site of recurrence appears to be determined by the location of the primary infection (Chang, 1971). The primary exposure of individuals to herpes simplex virus usually occurs early in life. Most of these infections are

TABLE 3
HERPES VIRUSES AS CANDIDATES FOR CLASSICAL LATENCY

Virus	Melnick's group	%G+C	Typical primary infection[a]	
			Local or widespread	Pulmonary involvement
B virus (Herpesvirus simiae)	A	75	L	-
Pseudorabies virus (Aujeszky's disease virus)	A	73	L-W	+
Spider monkey herpes virus[c]	A	71	L-W	+
Infectious bovine rhinotracheitis virus	A	71	W[b]	+
Squirrel monkey herpes virus[d] (Herpesvirus tamarinus)	A	67	L	?
Herpes simplex virus 2	A	68	L	-
Herpes simplex virus 1	A	66	L	-
Bovine mammilitis virus	A	64	L-W	-
Equine abortion virus	A	56	L-W[b]	+
SA8	A		L?	?
Varicella-zoster virus	B	46	W	+
Feline rhinotracheitis virus	B	46	W	+
Canine herpesvirus	B	33	L	+

A Viruses have a wide host range tissue culture and are not greatly cell associated
B Viruses have narrow host range and tend to remain cell associated
a In the natural host, immunocompetent, non-neonate
b Genital infection is markedly localized
c Not to be confused with Herpesvirus ateles
d Not to be confused with Herpesvirus saimiri

TABLE 3 (continued)

Natural host	Other experimental hosts	Recurrence or recrudescence	Reactivation from nervous tissue
Rhesus	(Man)rabbit guinea-pigs, other simians	+ usually lethal	+
Swine	Rabbits,calves mice and many other species	+ usually lethal	+
Ateles	Guinea-pigs, marmosets, rabbits	+? usually lethal	
Cattle	Rabbits, cotton tail rabbits	+	-?
Squirrel monkey	Mice,rabbits	+? +	+
Man Man	Mice, rabbits, guinea-pigs	+ + +	+ + +
Cattle	Mice,rabbits	+	-?
Horses	Hamsters,mice	+	-?
Vervet	Rabbit	+?	+
Man	None[e]	+	+
Cats		+	+
Dogs		+	-

e D-herpes virus of patas monkey (serologically related to varicella-zoster)
+? probably
-? tried but not yet successful
L localized
W widespread

FIGURE 6

DIAGRAMMATIC ILLUSTRATION OF HUMAN SENSORY GANGLIA (LUMBO-SACRAL REGION)

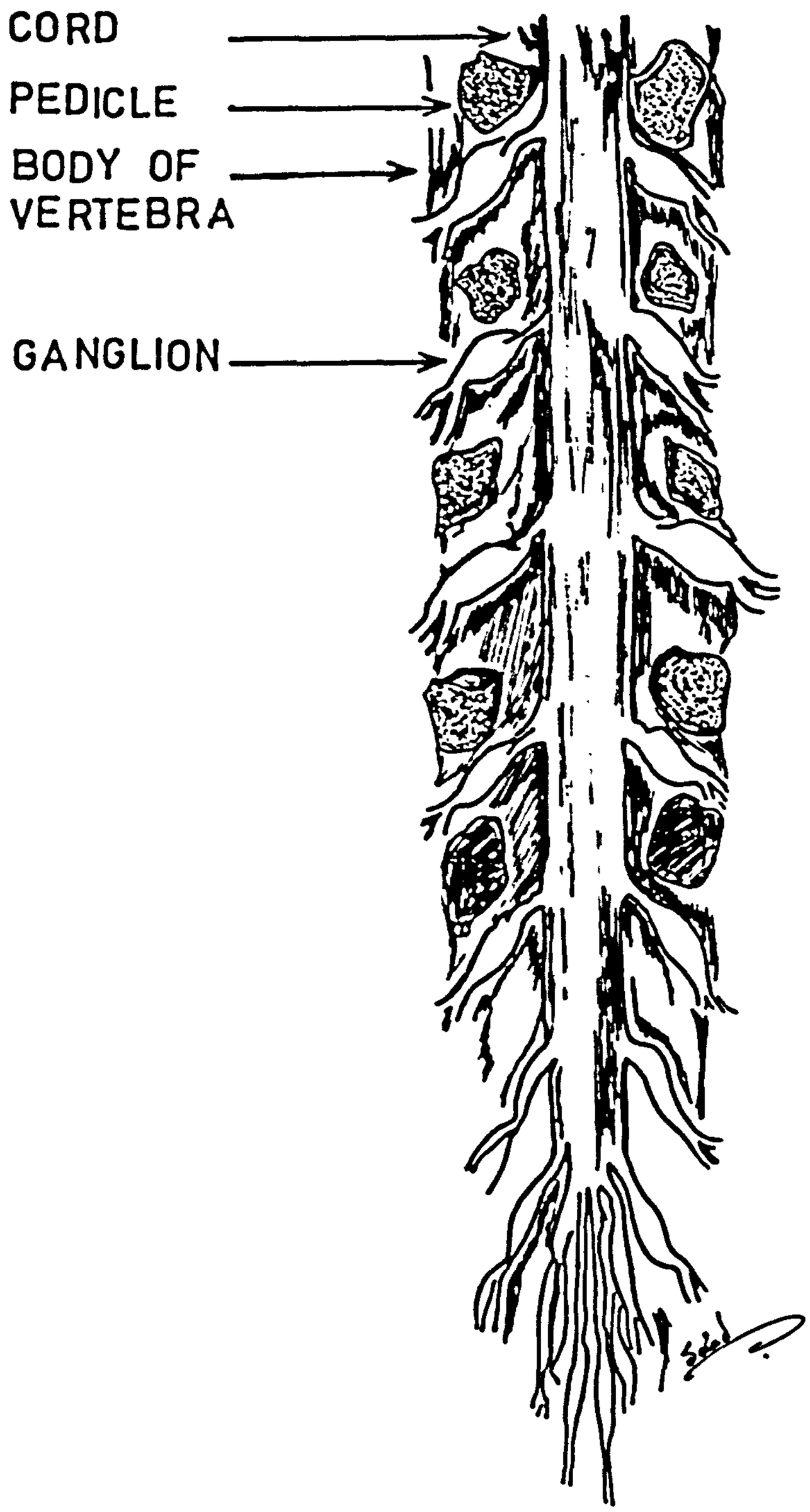
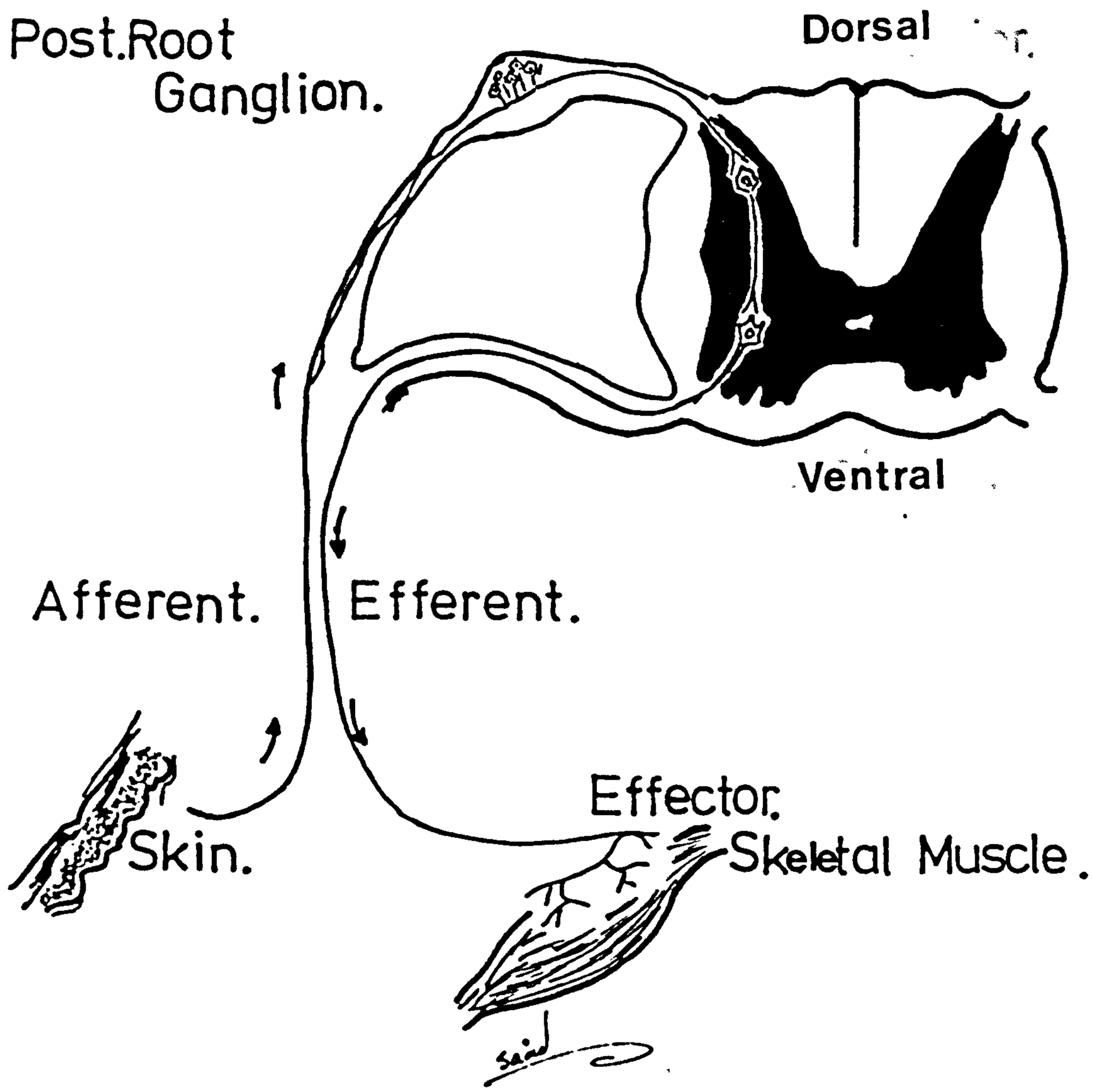


FIGURE 7

DIAGRAM OF RELATIONS OF SENSORY NEURONES (DORSAL ROOT GANGLIA) TO OTHER TYPES OF TISSUES



subclinical and the principal clinical manifestation is gingivostomatitis (Scott et al., 1941). The incidence of primary infection with herpes simplex virus type 2 increases with increasing sexual activity and a significant proportion of the adult population have antibodies to this strain of herpes simplex virus (Rawls et al., 1973; Nahmias et al., 1974). Recurrent herpes simplex has been recognised for centuries, and the early evidence which suggests that herpes simplex virus might remain latent within nervous system tissues and specifically within the trigeminal (TG) or other sensory ganglia of humans, was provided by a variety of clinical observations. These were: many patients can predict the appearance of vesicles because of a sensation of numbness, tingling or pain in the area 12-48 hours before the appearance of the eruption in the skin (Behrman and Knight, 1954; Layzer and Conant, 1974). In addition some factors are well known to have the ability to provoke recurrent herpes infections such as stress, allergic reactions, menstruation, fever, exposure to sunlight, trauma and sexual activities (Warren et al., 1940; Chang, 1971; Nahmias and Roizman, 1973a, b, c; Segal et al., 1974). Other evidence which also suggests the involvement of the nervous system particularly the trigeminal ganglia, in provocation of herpes simplex virus lesions, is provided by the observation that 90% of patients subjected to trigeminal rhizotomy develop herpetic lesions on their lips or mouth (Ellison et al., 1959). In fact, some years earlier, Carton (1953) showed that herpes lesions occur after section of the trigeminal nerve proximal to the ganglion, while section of the nerve distal to the ganglion will not lead to the development of such lesions. In fact, section of the nerve

distal to the ganglion or damaging the nerve by injection of alcohol may prevent herpes on the face. Moreover, herpes usually occurs only in the area innervated by the maxillary and mandibular branches of the trigeminal nerve and not the ophthalmic branches (Fig. 8). However, asymptomatic shedding of HSV in the tears has been observed presumably after reactivation of virus in the trigeminal ganglion (Klein, 1982). Furthermore, it has been suggested that latent HSV-1 may not be located only in neurones associated with the site of primary infection, which, in the majority of cases is the oral cavity and the perioral area - innerveted by the maxillary and mandibular branches (Klein, 1976). Recovery of herpes simplex virus from explanted human trigeminal ganglia (Barringer and Swoveland, 1973) has confirmed the belief that virus resides in the human trigeminal nerve ganglion during clinically inactive herpes.

In addition to the surgical manipulation of the trigeminal structures, other factors such as fever and sunburn (light) have been used to precipitate recurrent herpes in man. Fever was produced by using diathermy, infrared bulbs, blankets and hot water baths (Warren et al., 1940; Keddie et al., 1941). About 75% of fever treated patients developed herpes, and herpetic eruptions usually appeared within 24-48 hours after the induction of fever. The results suggest that almost everyone who has been infected with herpes virus can be made to develop recurrent herpes if the stimulus is strong enough. Evidence for the latter conclusion came from the observation that if the initial fever was between 39°C and 39.5°C about 55% of patients develop herpes, while increasing the initial fever to 40.8°C-41°C will result in herpes development in about

FIGURE 8

DIAGRAM ILLUSTRATING THE THREE BRANCHES OF HUMAN TRIGEMINAL GANGLION (TG)

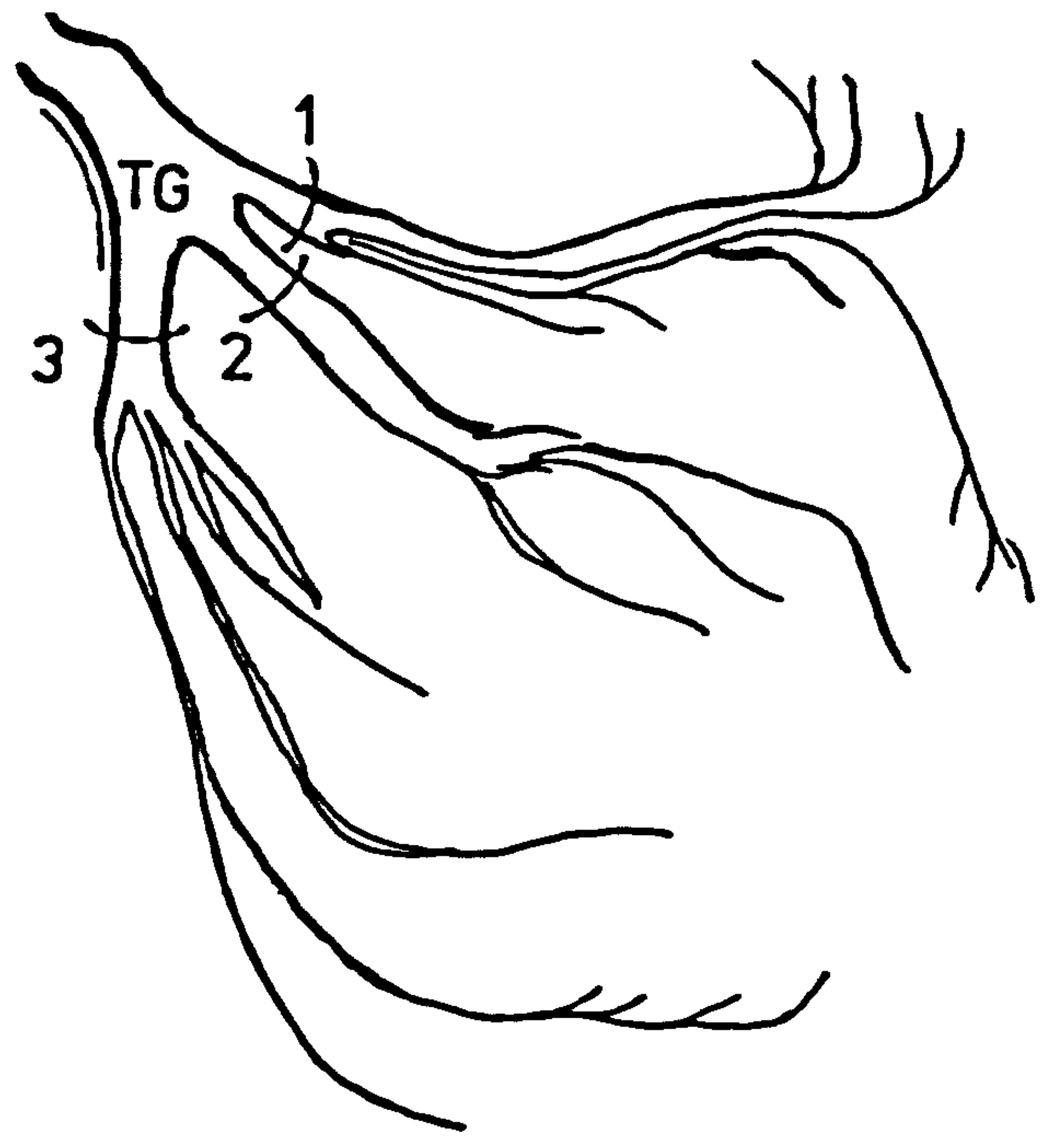
a) lateral view

b) superior view

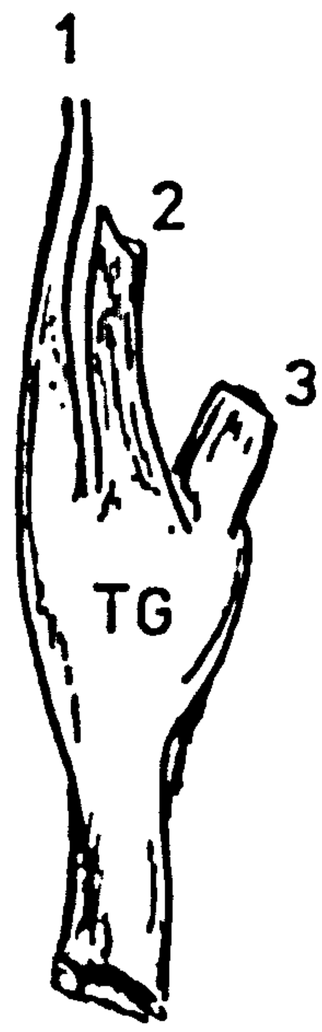
1. ophthalmic branch
2. maxillary branch
3. mandibular branch

Herpes lesions occurs after sectioning nerve proximal to the ganglion.

a)



b)



86% of the total patients (Keddie et al., 1941). Using sunburn to induce recurrent herpes (in patients known to have had herpetic infection) Wheeler (1975) suggested the administration of 8 minimal erythema doses of ultraviolet to a 3-inch diameter circle centered over a healed recurrent site. Herpes was precipitated in the three patients in four of nine attempts giving 4.5 to 8 minimal erythema doses of U.V. light. These and other reactivation inducing agents will be discussed later.

The data reported by Spruance et al. (1977) confirmed the previous observation about the short lived nature of lesions in recurrent herpes simplex labialis. The data showed that two thirds of the 80 patients studied, had prodromal symptoms which generally lasted a few hours only and that pain was experienced early, gradually reducing. Moreover, the progression "erythema -> papule -> vesicle -> ulcer -> healing" was usual and most lesions progressed from the vesicle to the ulcer stage within 48 hours; virus was isolated from 60% of patients with papules and from all patients with vesicles. No virus was isolated during the prodromal or erythematous stage, though virus was retrieved by cocultivation in 3/6 attempts. However, Daniels et al. (1975) were able to isolate virus, on several occasions, up to three weeks from recurrent lesions persisting in two immunocompromised patients. Infectious virus was frequently in the form of immune complexes.

Three main hypotheses concerning the location of the virus during the latent stage of infection have been described (Pain, 1964; Chang, 1971; Nahmias and Roizman, 1973a, b, c). The first hypothesis postulates that a chronic infection of the mucous membranes and glandular

appendages of the eye, mouth or genitalia is established and serves as a source of virus for clinically evident recurrences. Supporting this theory is the observation that virus is present in tears, saliva and genital secretions and can be isolated from time to time in the absence of clinical disease (Buddingh et al., 1953). Such virus can produce lesions in the cornea, lip, vulva and penile skin. However, this does not explain how virus from mucocutaneous areas can repeatedly produce recurrent lesions at distant sites e.g. on the buttocks, hands or lower legs. Moreover, asymptomatic shedding of virus in tears has been observed after microneurosurgical decompression of the trigeminal ganglion nerve root (Pazin et al., 1978). The second theory is that virus is present in some latent, incomplete or inactive, form at the site of recurrence, i.e. the skin and mucous membrane. The third theory, says that virus is present in some latent, incomplete or inactive, form in nerve tissue at a point distant from the recurrent site (e.g. sensory ganglia) which innervates the recurrent site. It is the latter two theories that attracted the attention of many workers and they will be discussed in detail later. However, it is relevant to discuss them from the natural host's point of view, in the following paragraph.

Human Skin Tissue as a Site of Latent Infection

It seems to be reasonable to consider that latent HSV resides in the skin during the interval between recurrences. According to one point of view, the site of latent infection is the skin or the mucous membranes where the primary infection occurred (Kauffman et al., 1967; Sabin, 1975). This idea is supported by the following facts: first, HSV has been isolated from the saliva and lachrymal secretions

of healthy carriers (Buddingh et al., 1953; Kauffman et al., 1968; Douglas et al., 1970). Second, 10% of non pregnant women with a history of recurrent herpes presented evidence of asymptomatic HSV shedding (Adam et al., 1979), while 70% of mothers delivering HSV infected newborns shed virus asymptomatically (Whitley et al., 1980). In addition, experimental reinoculation of the virus into human skin induces a new recurrence at the site (Blank and Haines, 1973). Furthermore, herpes simplex virus type 2 has been isolated from the buffy coat of two adults with meningitis (Craig and Nahmias, 1973). However, the idea that the skin is the site of latent infection has generally been hampered by the failure to demonstrate the presence of the virus (either by isolation from homogenized tissues or by organ culture) in the skin during the quiescent stage (Findlay and MacCallum, 1940; Rustigian et al., 1966; Wohlenberg et al., 1976).

Early studies in man (Stadler and Zurukzoglu, 1936) had shown that it was not possible to transfer a recurrence by switching skin grafts on the same patient, but recurrent lesions were observed in skin adjacent to the donor site, suggesting that recurrence did not depend upon residence of the virus in the skin. However, this was done in only two or three patients in the 1930s and it is hardly conclusive (Wheeler, 1975).

The observation that local ultraviolet light administration precipitates recurrent herpes (Wheeler, 1975) suggested that the virus may be in the skin, but it could alternatively be due to light (or heat) serving to call the virus forth from a nerve ganglion. However, as will be explained later, some evidence has accumulated suggesting

that latent infection can be induced (at least in experimental animals) in peripheral tissues (Scriba, 1977, 1981; Hill et al., 1980; Al-Saadi et al., 1983). Moreover, Hoyt and Bilson (1976) reported recurrent herpes simplex in patients with blow-out fractures which had severed the nerve supply to the area of skin involved, indicating that the lesions must have arisen from virus already present in the skin. Nevertheless, the skin is unlikely to be the most important site in view of the substantial evidence that reappearance of herpetic lesions is dependent on the preservation of intact anterior roots of viable ganglionic cells (Klein, 1976).

Neuronal Tissue as the Site of Latent Infection

The concept that the virus resides in the some latent or inactive form in nerves has been popular for years (Cushing, 1904; Howard, 1905). Goodpasture (1929) was the first to hypothesize the location of HSV in ganglionic tissue. This was supported by the clinical observation that herpes eruptions regularly followed rhizotomy (Cushing, 1904; Carton and Kilbourne, 1952; Carton, 1953). A further suggestion that nervous tissue is involved in recurrence of herpes simplex virus lesions comes from the observation that most of the patients experienced burning, itching and neuralgic pains 24-48 hours before the appearance of lesions (Behrman and Knight, 1954; Constantine et al., 1968; Layzer and Conant, 1974). However, while early attempts to isolate the virus from the ganglion were unsuccessful (Burnet and Lush, 1939; Ritchter, 1944; Carton and Kilbourne, 1952; Ellison et al., 1959), herpes simplex virus isolation from both the semilunar (Bastian et al., 1972; Baringer and Swoveland, 1973; Plummer, 1973; Rodda et al., 1973) and

sacral (Baringer, 1974) ganglia was successfully achieved during the past decade. Furthermore, herpes simplex specific RNA has been detected in sensory ganglia (Galloway et al., 1979) of patients with no evidence of herpes or recurrent infection, though no attempts were reported to recover the infectious virus and it is not clear if this hybridization was to neurones containing truly latent virus. In addition to the sensory or dorsal root ganglia other neurological sites have been speculated as possibilities for latent HSV infection. Warren et al. (1978) have demonstrated (by isolation) the presence of latent herpes simplex virus in the superior cervical and vagus ganglia of human beings. Furthermore, there is unconfirmed evidence for the presence of herpes simplex virus in the central nervous system (CNS) of man, using DNA hybridization (Sequera et al., 1979). Also there is a report of rescue of defective viruses from long term trigeminal ganglion cell cultures by superinfection with ts mutants at the non-permissive temperatures (Brown et al., 1979). The presence of HSV genomes in the CNS of man has been proposed as a possible precursor of encephalitis or it might provide a stimulus for demyelination. In general, it has been concluded that the sensory ganglion is the important site in man which harbours herpes simplex virus (see Table 3) in classical latency (Wildy et al., 1982).

Latent HSV Infection in Experimental Models

The different stages (initiation or establishment, maintenance, reactivation) of the mechanism of latent HSV infection have been variably investigated both in in vitro and in vivo experimental models. Among the experimental animals which have been used are mice (Stevens and Cook,

1971; Hill et al., 1975; Harbour et al., 1981; Al-Saadi et al., 1983), rabbits (Perdrau, 1938; Good and Campbell, 1948; Schmidt and Rasmussen, 1960; Nesburn et al., 1967; Plummer et al., 1970), guinea-pigs (Scriba, 1975; Donnenberg et al., 1980), and tree-shrews (Campbell, 1966; Goodman, 1966; Munk et al., 1978; Darai et al., 1978). The main point to note is that some aspects of herpes pathogenesis, latency and reactivation are reproducible in the animal models. Moreover, some of these models provide a close reproduction of the disease in humans: for example, recrudescence in mice (Hill et al., 1975; Harbour et al., 1981), recurrent genital herpes infection in guinea pigs (Scriba, 1975) and recurrent ocular herpetic infection in rabbits (Anderson et al., 1961; Nesburn et al., 1967). Beside, host strain differences (if any) can be studied in laboratory animals, particularly when a large variety of host strains are available e.g. mice used during this study. However, since the molecular aspects of HSV infection and latency are difficult to study in vivo, investigations have examined the interaction between HSV and some cells (both of neural and non-neural origin) in vitro. Moreover, using the latter system may help to shed light on the mechanism in man and in animals. Nevertheless it should always be kept in mind that human and animal infections involve additional complications due to the activity of their immune system.

In Vitro Studies

Many and varied cell culture systems have been used to study HSV-cell interaction resulting in long term infected cell lines and during the quiescent stage of infection different terms (chronic, prolonged, persistent and abortive) have been used to describe the nature of

infections. The goal of the investigations was to establish a reliable model system to study HSV latency in vitro.

Certain criteria have been suggested (Levine et al., 1980) for any HSV cell culture latency model: i, survival of infected cells, ii, absence of infectious particles in the surviving culture, iii, retention of the complete viral genome in the surviving cells, iv, ability to induce the synthesis of infectious virus by some manipulation of the culture. These criteria, however, do not take into account the fact that naturally occurring latent infection does not require as a prerequisite a certain alteration in the host body temperature, or the presence of viral inhibitors such as arabinosyl cytosine (ara-c) or phosphonoacetic acid which are used in most of the in vitro systems; moreover the criteria do not take into consideration the nature of cells harbouring latent virus under natural conditions, i.e. the non-dividing neurons (and perhaps other cells) that might be latently infected by viruses which have travelled from the primary site of entry. In addition, low levels of virus replication or virus gene expression (i.e. persistence) should be excluded as viral antigens can not normally be detected in latently infected neurones.

Some of the in vitro HSV cell interactions are listed in Table No.4. In reports where the infection is described as latent (O'Neill et al., 1972, 1977; Colberg-Poley et al., 1979, 1981; Wigdahl et al., 1982a, 1983), supportive measures (i.e. antimetabolites, interferon and elevation of the temperature of incubation) were essential in preventing virus replication. Moreover, infectious virus was detected within a short period upon removal of additives or upon lowering the temperature of incubation. Recently, it was

TABLE 4

SUMMARY OF SOME OF THE IN VITRO SYSTEMS

Virus	Type of Cell	Nature of Infection Described As:	Type of Supportive Measures	Reference
HSV-1	C6 rat glioblastoma	persistent	-	Rice *et al.*,1979 Dawson *et al.*,1983
	B103 rat brain neuroma	persistent	elevated temperature of incubation	Levine *et al.*,1980
	C143 rat CNS neuroma	persistent	-	Doller *et al.*,1979
	C-1300 mouse neuroblastoma	persistent	-	Vahlne and Lycke,1977
	Cells from rat sensory ganglia (purified neurone culture)*	latent?	BVDU[1] elevated temperature of incubation, IFN[2]	Wigdahl *et al.*,1983
	Raji P_3HR Burkitt lymphoma derived cell line (human)	persistent	-	Robey *et al.*,1976
	HEL human embryo lung	latent?	ara-c elevated temperature of incubation	Wigdahl *et al.*,1982a
HSV-2	C6 rat glioblastoma	abortive	-	Smith and Lancz,1982
	C-1300 mouse neuroblastoma	persistent	-	Vahlne and Lycke,1977
	Raji Burkitt lymphoma derived cell line	persistent	-	Robey *et al.*,1976
	HEL human embryo lung cell	latent?	ara-c	O'Neill *et al.*,1972,1977
	HFF human fetal fibroblast	latent?	phosphonoacetic acid elevated temperature	Colberg-Poley *et al.*, 1979,1981

1 E-5-C-2-bromovinyl 1-2-deoxy-uridine
2 human leukocyte interferon
* ara-c fluorodeoxyuridine and uridine (antimiotic agents) were used to prepare the culture

possible to establish a similar type of latent infection in C13 cells without the presence of supportive measures (E. Notarianni, personal communication). In another study Wigdahl et al. (1983) have claimed the establishment of latent HSV-1 infection in neuron culture, but the presence of infectious virus was detected within 2-3 days after lowering the temperature of incubation and the presence of contaminating fibroblast and Schwanncells in the culture was not excluded. It can be seen that the conditions under which the infection was established are highly artificial and in none of these systems has a true latent infection been established that can be said to mimic the course of the infection in vivo. However, it is worth discussing HSV cell interaction in some of these systems.

Different studies had shown that cells range from non permissive, semi permissive to permissive for HSV replication (Schwartz and Elizan, 1973; Vahlne and Slycke, 1977, 1978; Adler et al., 1978; Doller et al., 1979; Lewin et al., 1980; Smith and Lancz, 1982) and that HSV can persistently infect a variety of cells (Table No.4) including neural and non neural (i.e. epithelial, fibroblast and lymphoid) cells of different species, e.g. Chinese hamster ovary cells (Hampar and Copeland, 1965; Hampar and Burroghs, 1969), L cells (Nii, 1969), HeLa cells, (Szanto, 1963) both in the presence or absence of supportive measures. In most of these cells, however, there is lack of evidence for retention of HSV genetic information in cultures that are no longer producing infectious virus.

Results of these studies have indicated that persistent HSV infection can be maintained for variable often prolonged lengths of time, or as long as the cultures

were maintained under supraoptimal temperature e.g. for 128 days in HFF cells (O'Neill et al., 1977). It was also shown that in these cells (B103) non permissiveness is temperature and multiplicity dependent (Lewin et al., 1980). On the other hand, it has been shown (Rice et al., 1979) that in contrast to human glioma (COX) and mouse neuroblastoma (D2) cells, rat glioma (C6) cells do not support generalised HSV-1 replication and a persistent infection of these cells (i.e. C6) is established. In conclusion, HSV replication patterns in cultured cells are quite variable and no generalization can be made particularly for HSV infection in neural cells.

As in latency, the molecular basis of the persistent infection is unclear, and the state of the virus genome in such types of infections is not understood. Manipulations of persistently infected cell cultures (lowering the temperature or removal of inhibitors) have resulted in resumption of virus growth and both released virus and cpe were observed in the treated culture (Rice et al., 1979; Dawson et al., 1983). Stimulation of HSV-2 production in persistently infected cultures was also achieved by superinfecting cells with the wt virus and ts mutants of human cytomegalovirus (Colberg-Poley et al., 1979, 1981). Similarly, superinfection with HSV-2 ts mutants stimulated HSV-1 genome in persistently infected cell culture (Wigdahl et al., 1982; Park and Macnab, 1983). Evidence in both of the superinfection systems indicated that HCMV and HSV-2 DNA synthesis is not required for activation of repressed HSV genomes suggesting the involvement of early genome functions in the stimulation process. However, it is not clear whether the superinfection mechanism stimulates cellular

functions that are required for HSV replication, or directly stimulates virus transcription, or alternatively, provides functions or products necessary for replication and there is no evidence to support any of the latter possibilities. In addition, recombination between repressed HSV-1 and inoculated HSV-2 genomes has not been totally excluded (Wigdahl et al., 1982).

It has been reported that some cell cultures persistently infected with HSV underwent a series of "crises" of cell destruction and regrowth, i.e. cyclical persistent infection (Hampar and Copeland, 1965; Nii, 1969; Robey et al., 1976; Szanto et al., 1976; Rice et al., 1979). Several models have been proposed to explain persistence and cyclical activity in tissue infected with cytopathic viruses (Friedman and Ramseur, 1979) including i, defective interfering (DI) particles, ii, involvement of the interferon system, iii, genetic changes of virus or host, e.g. spontaneously generated temperature sensitive mutants and iv, probably integrated DNA. Though a proportion of the Epstein-Barr virus DNA (human herpes virus) was found as an integrated form (Nonoyama and Pagano, 1972; Tanaka and Nonoyama, 1974) in the Raji cell line and subsequently in other EBV carrying lines, there is no evidence for integration of the HSV genome in persistently infected cells. The integration of EBV-DNA in host cells will be discussed later. The first two possibilities (DI particles and interferon) are subjects of controversy. The role of DI particles has not been confirmed and it has been proposed (Roizman, 1965) that both functional and defective subunits may play a role in the mechanism of HSV abortive infection. In addition, a low level of HSV DNA synthesis was observed

in C6 cells persistently infected with HSV-1 (Rice et al., 1979) which might preclude the formation of DI particles. However, this has not been confirmed since viral DNA synthesis and specific antigens were not detected in similar cell cultures infected with HSV-2. On the other hand, it has been shown that interferon is not involved (Lancz and Zettlemoyer, 1976; Smith and Lancz, 1982) or at least not of primary importance in the establishment and/or maintenance of persistent infection in C6 cells (Dawson et al., 1983).

Investigations attempting to characterize a HSV-cell interaction have produced inconsistent results, for example, certain variants of glioma cell lines vary in their ability to produce virus (Rice et al., 1979). Moreover a discrepancy between the levels of resistance and HSV production in C6 cells has been noticed in different reports (Schwartz and Elizan, 1973; Lancz and Zettlemoyer, 1976; Ando and Arai, 1980; Smith and Lancz, 1982; Dawson et al., 1983). These discrepancies probably explain the inconsistency of terms used to describe the infection (prolonged, chronic, persistent, abortive and others). These observations, indicate the important role of the virus and host cell factors in the mechanism of persistent infection.

In Vivo Studies

There is general agreement on the basic features of HSV infection. The virus enters the host organism in most cases through the mucocutaneous surface where the primary infection occurs then there is usually infiltration of the corresponding ganglia by the virus. For some individuals, the virus may periodically reactivate subsequently with recrudescence or without the development of detectable

lesions at the peripheral site within the infected dermatome (recurrence) (Wildy et al., 1982). It is convenient to review the mechansims of latency in the order of 1st, acute ganglionic infection; 2nd, establishment and maintenance of latent infection; 3rd, reactivation.

Acute Ganglionic Infection

Evidence for the presence of HSV virus in the spinal ganglia of mice, during the acute phase of infection, has been provided by Hill et al. (1972) and Cook and Stevens (1973). Following peripheral inoculation of mice, HSV can be demonstrated in spinal ganglia within 20-24 hours p.i. The amount of infectious virus that can be detected in the corresponding ganglia increases for the first 4-7 days p.i. and then subsequently declines. The increase in the amount of virus in the ganglia leads to the assumption that a productive neuronal infection occurs during the acute phase of the infection (Cook and Stevens, 1973). Supporting this and the assumption that HSV travels centripetally along the axon, is the observation of the presence of virions in neuronal bodies and axons several days after infection (Wildy, 1967; Hill et al., 1972; Cook and Stevens, 1973; Knotts et al., 1974; Kristensson et al., 1974, 1978). Moreover, the presence of viral mRNA in the ganglia during the acute phase of infection has also been demonstrated indicating a productive infection (Puga et al., 1978). However, the viral RNA:DNA ratio suggests that either virus DNA replication was limited to only a few genomes or the lytic cycle was arrested during an early stage of infection. In addition, topical treatment of the inoculation site with 2% phosphonoacetic acid (PAA) resulted in suppression of virus multiplication at the site of inoculation with

subsequent drop of virus titre in corresponding ganglia (Klein and Destefano, 1981). This indicated that local antiviral treatment could reduce the viral titre in ganglia by halting the continuous virus supply from the site of primary infection. However, the possibility that productive ganglionic infection may occur to a limited extent has not been totally excluded. On the other hand good evidence has indicated that a productive infection is not necessary for the establishment of latent infection in neurones or ganglia. Using HSV-1 temperature-sensitive mutants, Lofgren et al. (1977) showed that there is no apparent relationship between the capacity of the mutant to induce encephalitis and the subsequent establishment of latent infection in brains of mice. Similarly, McLennan and Darby (1980) have concluded that there is no absolute requirement for a productive infection during the establishment of latency in the neurone. Furthermore, it has been shown that acyclovir resistant (ACVr) mutants can establish acute but not latent infections in trigeminal ganglia of mice (Klein et al., 1980).

Virus Pathway to the Nervous System

The intra-axonal transport of herpesvirus was proposed in the early years of this century (Goodpasture, 1929). Four alternative routes have been suggested, namely, virus travels within the periaxonal (extracellular) spaces (Payling Wright, 1953); virus travels by multiplying in Schwann cells in a stepping-stone fashion along the nerve fibres (Johnson, 1964); virus travels through lymphatics or as a viraemia (Field, 1952). Evidence against the possibility that the virus travels through periaxonal spaces or by multiplication in supporting (Schwann) cells, is

provided by the demonstration of virus first in the ganglia and spinal cord and only afterwards in the sciatic nerve (Wildy, 1967; Kristensson et al., 1971; Cook and Stevens, 1973). Moreover, virus titres were consistently higher in ganglia than in the sciatic nerve (Cook and Stevens, 1973; Klein, 1982). In addition, viruses were almost never seen to be free in the extracellular space and were only rarely seen in supporting cells. On the contrary, intact virions were observed within the axoplasm, though it is not possible to be certain that the particles observed are the effective virions.

The failure to detect HSV virus in the distal and proximal sections of the sciatic nerves (during the first 2 days p.i.) and the fact that HSV is first detected in the ganglia and only afterwards in nerves, suggested an alternative (haematogenous or lymphatic) route may be involved in the dissemination of virus to the nervous system. Cook and Stevens (1973) have tentatively concluded that since HSV infection progresses (i.e. the development of lesions in the nervous system) despite the presence of significant levels of neutralizing antibody (passive administration of anti-viral sera), blood and lymph vessels are not involved in the transport of virus to ganglia. However, following the inoculation of the virus in the footpad of guinea pigs, HSV-2 has been regularly found in the regional lymph nodes by the second day p.i. (Scriba and Tatesber, 1981). Some other studies (Kristensson et al., 1971; Knotts et al., 1974; Renis et al., 1976; Kristensson et al., 1978) also reported the absence of herpes simplex virus in blood following corneal, footpad, intravaginal or intraperitoneal inoculation. But HSV has been detected in

the blood of newborn and adult mice following footpad (Wildy, 1967; Lascano and Berria, 1980; Klein, 1982) or intranasal inoculation of newborn mice (Kern et al., 1975). It is not known whether virus invades the nervous system subsequent to viraemia or whether viraemia is due to virus spill over from the peripheral nervous tissues. Direct evidence for the possible involvement of the circulatory system is the demonstration that HSV-1 selectively establishes latent infection in mouse neural tissue, particularly the sensory ganglia, after intravenous inoculation. This was shown conclusively when (tail vein) inoculation was immediately followed by tail amputation at 2.5 cm proximal to the injection site (Cook and Stevens, 1976). It is possible that nervous tissue may not be infected directly by virus in the blood stream, but indirectly by virus travelling in the nerves associated with vessels or other organs that became first infected through viraemia.

Using the PAP method (peroxidase antiperoxidase) to trace viral antigen, Lascano and Berria (1980) reported evidence that supports the mixed route for the neural transport of HSV-1 in newborn mice. This study suggested that the neural transport of HSV depends on more than one mechanism: a. cell-to-cell active transport (Schwann and connective tissue cells of the sciatic nerve) b, passive movement of the virions along the intracellular spaces within the neural structures.

It is obvious that much controversy persists concerning the mechanism of virus transport. Another approach (neurectomy) has been used to investigate the possible structures involved in dissemination of virus to

the nervous system. Wildy (1967) showed that sciatic nerve sectioning three days before virus inoculation, prevented HSV isolation from the spinal cord in 80% of mice. Moreover, Kristensson et al. (1971) reported that colchicine treatment, ligature or freezing of sciatic nerve 0-4 days before virus inoculation into the footpad of mice reduced the mortality rate to 10% compared to 100% if the treatment was carried out after inoculation. In neither of the above reports were attempts made to recover the virus from ganglia. Total surgical section of the sciatic and femoral nerves completely prevented the establishment of latent HSV infection in sensory ganglia of guinea-pigs inoculated into the footpad (Scriba, 1981). Similar experiments in the mouse (Klein, 1982) revealed that establishment of acute infection in spinal ganglia was prevented by section of both femoral and sciatic nerves in about 80% of mice. Free virus, however, was regularly demonstrated on the 4th and 7th day after infection in spinal ganglia of mice in which either the sciatic or femoral nerves were severed. Further evidence supporting the axonal transport hypothesis has come from the in vitro study of attachment of HSV to rabbit and rat astrocytic glia, neuronal perikarya and (nerve terminals) synaptosomal fractions (Vahlne et al., 1978). HSV preferentially adsorbs to the synaptosome fraction rather than neuronal perikarya. However, it remains unclear whether this difference in virus attachment was due to the presence of specific HSV receptors on the synaptosomes or merely reflected a high endocytic activity of the synaptosomes.

Another observation is the rate of HSV translocation from the skin to the ganglia which has been reported to be

in agreement with the rate of movement of macromolecules and organelles by retrograde axonal transport (Cook and Stevens, 1973; Kristensson et al., 1974, 1978).

In conclusion, it seems that axonal transport is the most important method of virus translocation, though the virus may also travel by other routes. Irrespective of the way virus travels to the nervous system, the subsequent result is almost always the establishment of latent infection.

Latent Phase of the Infection

Establishment

After the initial increase, the amount of detectable HSV in cell free ganglion homogenates decreases gradually; within 14 days p.i. virus can no longer be detected in homogenates, but HSV can still be recovered by explantation or cocultivation of ganglia. At this point the acute phase is terminated and the latent phase of the infection begins (Openshaw et al., 1979a; Sekizawa et al., 1980; Klein, 1982). The transition from the 'acute' to the 'latent' phase of infection, coincides with the appearance of immune responses, particularly high levels of antibodies. It has been proposed that immune factor(s) play a major role in the switch from the acute to the latent phase of infection (Openshaw et al., 1979a; Sekizawa et al., 1980; Openshaw et al., 1981), though the mechanism by which these immune responses could produce the transition from the acute to the latent state of infection is not clear. Some hypothetical models (Openshaw et al., 1981) have been proposed (Fig. 9): 1, The immune modulation model; this assumes that ganglion cells are permissive but the immune response modulates the infection at the level of the ganglion, converting a

FIGURE 9a

IMMUNE ELIMINATION MODEL FOR ESTABLISHMENT OF LATENCY

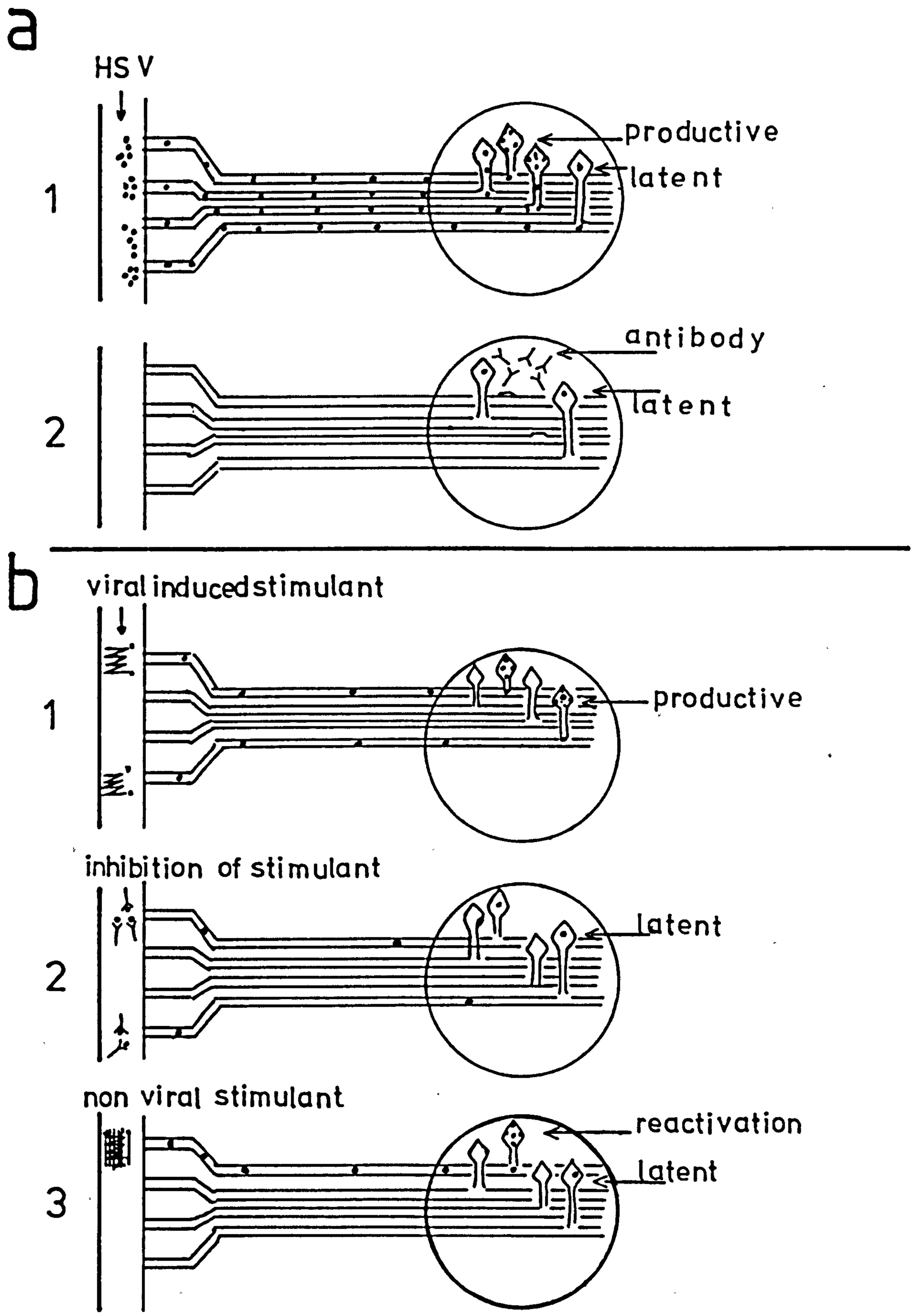
There are two populations of ganglion cells, one permissive and the other non-permissive for HSV replication.

1. latent infection is established in non-permissive cells while a productive infection develops in permissive cells
2. immune response aids in eliminating the productively infected cells leaving the latently infected cells intact

FIGURE 9b

NON-PERMISSIVE MODEL FOR LATENCY AND REACTIVATION

1. ganglion cells are primarly non-permissive for HSV replication. Replication of virus at the skin and/or the ensuing inflammation, directly or indirectly, provides a signal (stimulus) that makes the ganglion cells permissive for HSV
2. during the course of the natural infection, the host's immune response shuts off this signal by decreasing replication of the virus at the epithelial surface. This puts ganglion cells back to the non-permissive state and latency ensues
3. reactivation occurs when any non-viral stimulant, e.g. trauma, provides a signal at the epithelial site innervated by the latently infected ganglion cell. This makes the non-permissive ganglion cell once again permissive for HSV replication
   Openshaw *et al.*, 1981


a
HS V
1
productive
latent
2
antibody
latent
b
viral induced stimulant
1
productive
inhibition of stimulant
2
latent
non viral stimulant
3
reactivation
latent

potentially lytic infection into non-lytic or latent infection (i.e. the immune response plays an active role in establishing latency). 2, The immune elimination model, (Fig. 9a) where there are two populations of ganglion cells: permissive and nonpermissive. A productive HSV infection occurs in the permissive cells and a non lytic or latent infection occurs in the nonpermissive cells. The host immune response strictly has nothing to do with the establishment of latency, but just eliminates only acutely infected cells. 3, The third model (Fig. 9b) assumes, that ganglion cells are ordinarily nonpermissive for HSV replication, however they become permissive after receiving signals (induced by the inflammatory reaction in the skin due to virus replication). The host immune response then turns off these signals by decreasing virus replication in the skin, returning ganglion cells to the nonpermissive state hence latency is established. The above three models, rely solely on immune mechanisms to account for the transition from the acute to the latent state of infection. Another model has been proposed by Klein (1982) which assumes that ganglion cells are nonpermissive; colonization of the ganglia is achieved by continuous virus supply from the site of primary infection. Immune responses restrict virus replication in mucocutaneous tissue. It is suggested that the majority of virions in neurones are inactivated by cellular enzymes and only a minority will be in a latent form in cellular structures (Fig. 10).

It must be emphasized, that the above models are merely hypothetical and there is no hard evidence to support them. Besides, the models do not consider the possible establishment of latent infection in tissues other than

FIGURE 10

HYPOTHETICAL MODEL FOR THE TRANSITION FROM THE ACUTE TO THE LATENT PHASE OF HSV INFECTION

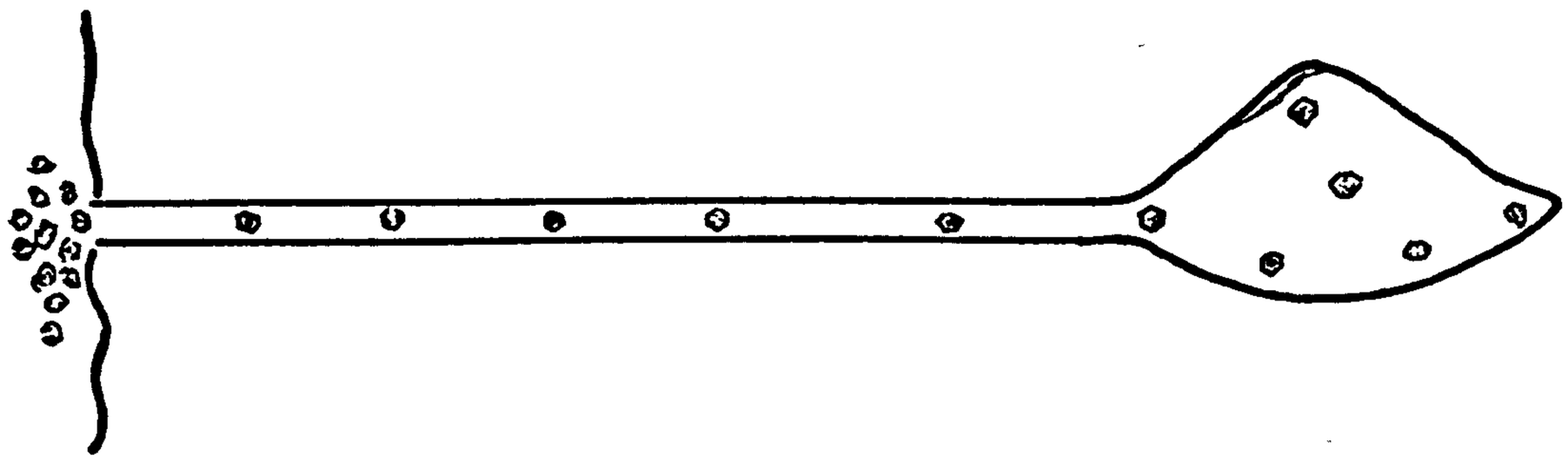
a) virus replicates in the skin then migrates to the corresponding ganglion (neurone)

b) development of immune responses, followed by neutralisation of virus in the skin which ends the process of virus migration

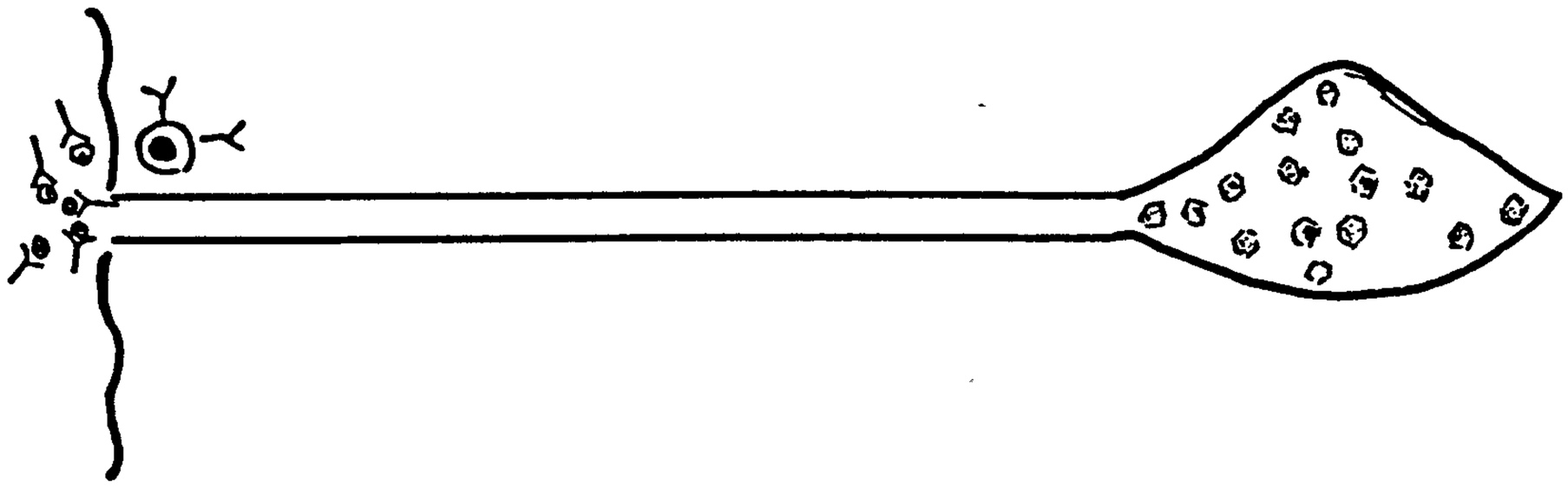
c) virus is eliminated from the skin. Immune and/or other mechanisms eliminate the majority of virus from neurones. A few virions remain latent within the cell

Klein, 1982

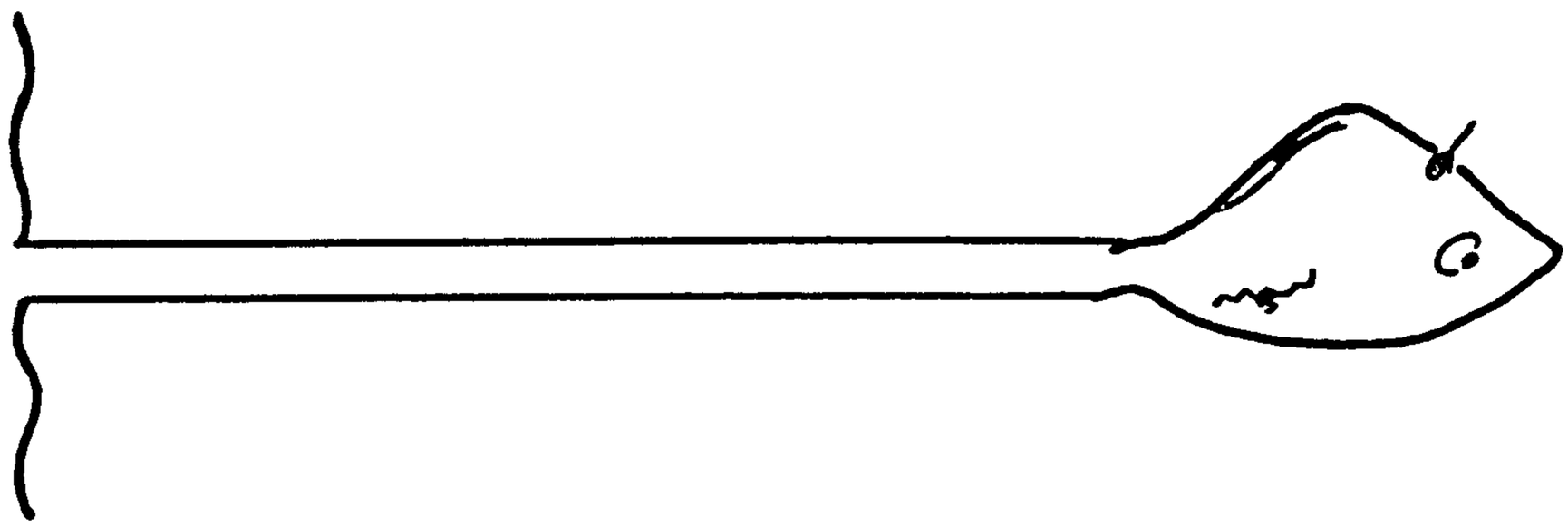
a



b



c



ganglion cells.

Maintenance

The factors involved in maintenance of HSV latency have not been fully demonstrated and no settled conclusions have been achieved yet. Stevens and Cook (1974) claimed that passive administration of anti-HSV IgG prevented reactivations; latently infected ganglia were less likely to reactivate when implanted (in a Millipore chamber) within the peritoneal cavity of immune mice than in non immune mice. However, this study did not distinguish between a direct effect of antibody on the reactivation process and a reduction in the ability of reactivated virus to subsequently replicate. Studies with immunised and nude mice showed that the immunological status of the host is one of the factors that controls the number of cells in ganglia that become infected (Walz et al., 1976). However, in vitro studies (Openshaw et al., 1979; Wohlenberg et al., 1979) revealed that neither antibody nor interferon treatment prevented reactivation. Furthermore, Sekizawa and co-workers (1980) were able to establish latent HSV infection in mice passively immunized with rabbit anti-HSV serum administered at various times post infection. A few months later, neutralizing antibody was undetectable in passively immunized mice which remained latently infected (i.e. ganglionic homogenates were negative but ganglionic explants yielded virus). Persistence of virus in the absence of neutralizing antibody suggests that once latency is established, serum neutralizing antibody does not appear to be required to maintain the latent state (Openshaw et al., 1981). However, a role for non neutralizing antibodies has not been excluded (Nash, 1981). Furthermore, anti-HSV

antibody found in cerebrospinal fluid (CSF) of acutely infected mice was probably not from the serum but possibly produced locally (Kastrukoff et al., 1981). Although the immune responses are thought to play a role, some other determining factors have been suggested as being involved in the prolonged maintenance of latent herpes simplex virus infection in sensory ganglia (Klein, 1982) of infected individuals:-

i) The number of latently infected neurones after termination of the acute phase.

ii) The number of neurones involved during each reactivation event.

iii) The fate of neurones after reactivation.

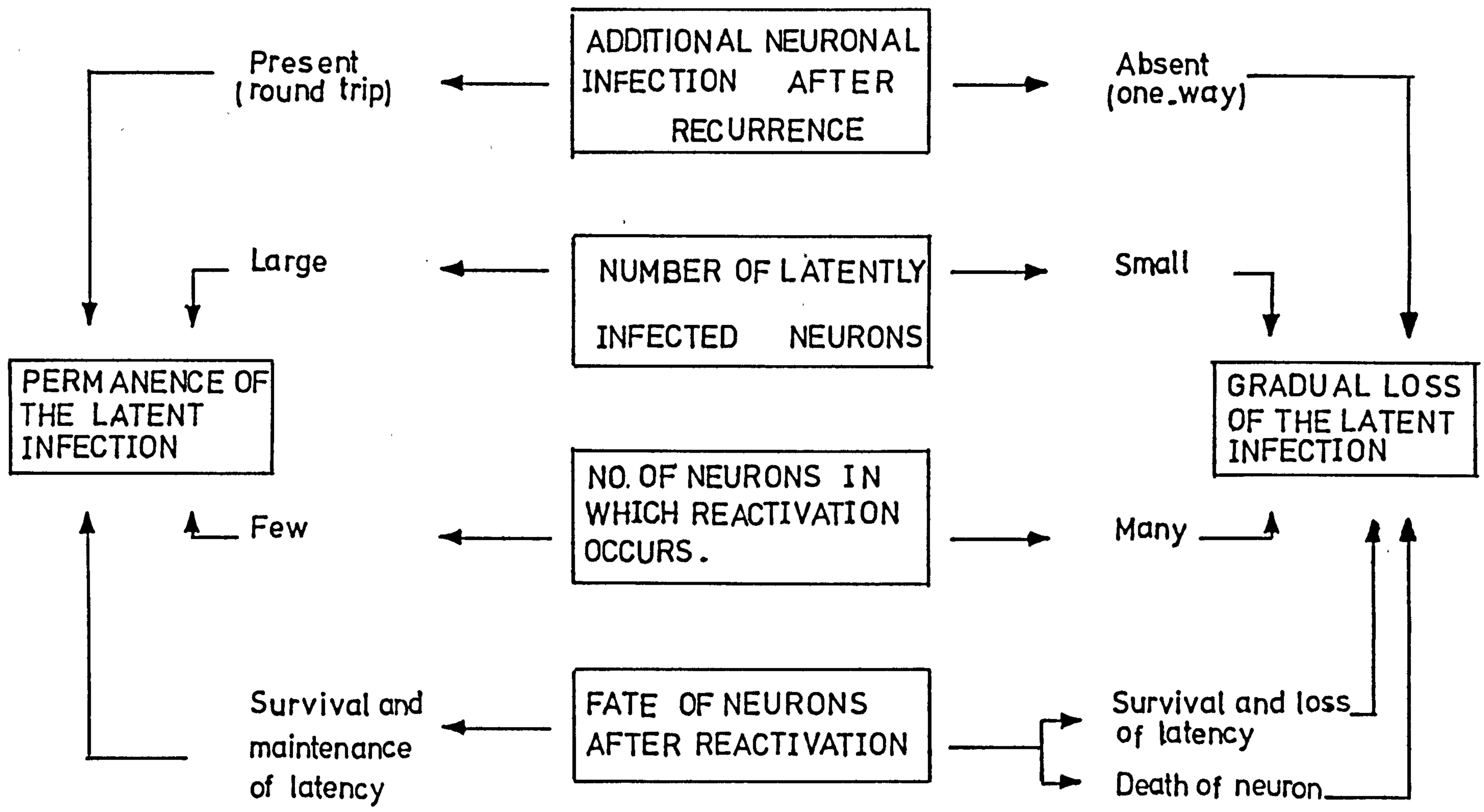
iv) The possibility of infecting new sets of neurones after each recurrent episode.

A schematic illustration of the above possibilities is presented in Figure 11.

An independent estimate of the total number of cells in the mouse trigeminal ganglion is not available, and the ratio of nonneuronal to neuronal cells is also unknown. However, the number of neurones in the trigeminal ganglion of man, cats and rats range from 50,000 to 124,000 with an estimated nonneuronal:neuronal ratio of 10-20 to 1 (Puga et al., 1978). Based on their relative numbers in the above species, the total number of cells in the mouse trigeminal ganglion was estimated as about $8x10^5$, with an average of 10^4 to $2x10^4$ neuronal cells per ganglion (Puga et al., 1978; Klein, 1982). If these figures are correct, then the estimate of the percentage of latently infected cells (by infectious center assay) in mouse trigeminal ganglia as 0.1% (10 to 20 neurones) is probably an underestimate (Walz et

FIGURE 11

QUANTITATIVE AND QUALITATIVE FACTORS INVOLVED IN THE PROLONGED MAINTENANCE OF LATENT HSV INFECTION IN SENSORY GANGLIA


ADDITIONAL NEURONAL INFECTION AFTER RECURRENCE
Present (round trip)
Absent (one-way)
NUMBER OF LATENTLY INFECTED NEURONS
Large
Small
PERMANENCE OF THE LATENT INFECTION
GRADUAL LOSS OF THE LATENT INFECTION
NO. OF NEURONS IN WHICH REACTIVATION OCCURS.
Few
Many
FATE OF NEURONS AFTER REACTIVATION
Survival and maintenance of latency
Survival and loss of latency
Death of neuron

al., 1976), due to possible loss of cells during physical manipulation and also because of the potentially low plating efficiency of infected cells. The proportion of latently infected neurones in the human has been assumed to range between 0.1 and 10 percent, i.e. 150-15,000 latently infected neurones in each trigeminal ganglion (Klein, 1982). The number of neurones in which reactivation takes place during each recurrent episode is unknown. However, experiments in which ganglia from latently infected mice (with HSV-1 wt and ts mutants) were excised 2 weeks after neurosurgery and then reactivated in vitro, have revealed a signficant reduction in the total number of resulting foci (McLennan and Darby, 1980). Moreover, the virus produced on culture of positive ganglia (of surgically manipulated mice) was significantly less than that generated in the controls. This would suggest that virus reactivation occurs in most or all latently infected neurones and that the consequence of reactivation is destruction of latently infected cells (neurones). However, the possibility that latency disappears (due to virus reactivation) only from those ganglia in which few neurones have been latently infected has not been excluded. Supporting this argument is the observation that virus can still be detected in 25% (McLennan and Darby, 1980) and in 32-62% (Klein, 1982) of ganglia after neurectomy. Furthermore, since preliminary data suggest that there is a relationship between the frequency of persistent infections after neurectomy and virus dose used in primary infection, it seems possible that ganglia in which large numbers of neurones were latently infected can continue to shed virus even after neurectomy.

It has been proposed that the productive replication

which follows activation of latent virus in the neurones leads to the death of that cell. Supporting this interpretation is the strong immunofluorescence observed in ganglion cells (neurones) following HSV reactivation (McLennan and Darby, 1980). Moreover, electron microscopic study revealed active virus synthesis in neurones of latently infected ganglia (rabbit trigeminal ganglia) maintained in culture (Barringer and Swoveland, 1974). However, in man, on the basis of clinical data, it is not clear whether the neurone is necessarily destroyed after an HSV reactivation episode and whether the latent virus may disappear from the cell after reactivation.

Klein (1976) has proposed two mechanisms, viz, "one-way" transmission and the "round-trip". In both mechanisms it is assumed that the neurone is not destroyed and reactivation occurs in all latently infected neurones. The "one-way" trip proposed that the number of latently infected neurones is fixed, and the virus is reactivated from the neurone, travels to the skin. However, the information for renewed recurrence is never lost from latently infected neurones. The "round-trip" proposed that infection of a new set of neurones is possible. After reactivation, some virions escape the elimination process at the neuronal level, and travel to the skin where they produce lesions. Then virus synthesized in the skin will migrate toward the neurone and re-establish a latent infection. Neither of these mechanisms has been proven or rejected; nevertheless since the reduction in frequency of latent foci was seen in mice infected with the most pathogenic HSV-1 strain (SC16 virus, Hill et al., 1975), it would seem unlikely that latency is re-established by spread

of reactivated virus within the ganglion (McLennan and Darby, 1980).

Virus State During Latency

Two hypotheses have been proposed by Roizman (1965), namely the "static-state" hypothesis which proposes that certain (virogenic) cells are capable of sheltering a non-replicating HSV or its genome between recurrent attacks; and the "dynamic state" hypothesis, where latent virus appears to be multiplying in a very slow fashion resulting in a chronic subclinical infection. Supporting evidence to the latter hypothesis, is the isolation of both HSV-1 (Buddingh et al., 1953; Lindgren et al., 1968; Douglas and Couch, 1970) and HSV-2 (Jeansson and Molin, 1970; Centifanto et al., 1971, 1972; Masukawa et al., 1972) from apparently healthy individuals during the quiescent stage of infection. Moreover, Kaufman et al. (1967, 1968) were able to isolate HSV from lachrymal glands of rabbits and from tears of humans. Furthermore, in vitro studies where persistent infection has been established in cell cultures, revealed cycles of cell destruction followed by periods of cellular recovery and repopulation (Hampar and Burrough, 1969; Nii, 1969, 1970). Those studies and those of others (Hampar and Keehn, 1967) underlined the role of the antibody and of genetically determined factors in the maintenance of the dynamic state of virus-cell interaction. On the other hand, nonspecific or specific factors may be involved in preventing the initiation and maintenance of virus multiplication (i.e. static state). It has been suggested (Roizman, 1965) that virus infected cells may specifically produce a substance (possibly interferon or some other product) that inhibits virus replication. It has also been

reported that extracts from Burkitt's lymphoma (Rabson et al., 1971) and from hamster cells transformed by HSV-2 (Doller and Duff, 1974) can inhibit the replication of herpes simplex virus. More evidence, supporting the static-state hypothesis is the demonstration of the presence of infectious HSV virus in ganglia of latently infected mice and rabbits, but only after placing the ganglia in organ culture (Stevens and Cook, 1971, 1973). Furthermore, in vitro studies (O'Neill et al., 1972) where persistent HSV-2 infection was established in HEL cells in the presence of ara-c, revealed that some cells contained HSV-2 in a non infectious form during the latent period (the period between the disappearance and subsequent reappearance of the virus). However, infectious HSV-2 reappeared several days after the removal of the inhibitors. Hence, following the prevention of synthesis of detectable infectious virus (by the inhibitors), HSV remains stable and is not degraded by the host cell (O'Neill et al., 1972).

The form HSV assumes while in a non replicating stage is unknown. However, the virus may possibly exist as a, complete virion present within a cell; b, incompletely assembled virions; c, nucleoprotein; d, solely as nucleic acid; e, nucleic acid with limited transcription and gene expression. No direct evidence is available favouring any one of the above possibilities as far as HSV is concerned. If the virion really exists as such and is fully infective within the cells, it should have been detected by homogenisation experiments or by hybridisation studies. If one assumes that virus exists as nucleic acid, it would be important to find out whether it exists in an integrated state (covalently linked to the host chromosomal DNA) or in

a nonintegrated state, i.e. possibly as an episomal factor (Docherty and Chopan, 1974). Strong evidence has been provided for the existence of the Epstein-Barr virus (EBV) genome within the nucleus of "virus-free" malignant lymphoma cells (Nonoyama and Pagano, 1972; Lindahl et al., 1976). But there are important differences between EBV and HSV induced infections, as the viruses interact with two different cell types (i.e. B-lymphocytes and neurones) within the individual host and as EBV "latent" infection has not been defined yet. Moreover, it seems unnecessary for the HSV genome to be integrated into the host cell since neurones do not divide (Fenner et al., 1974).

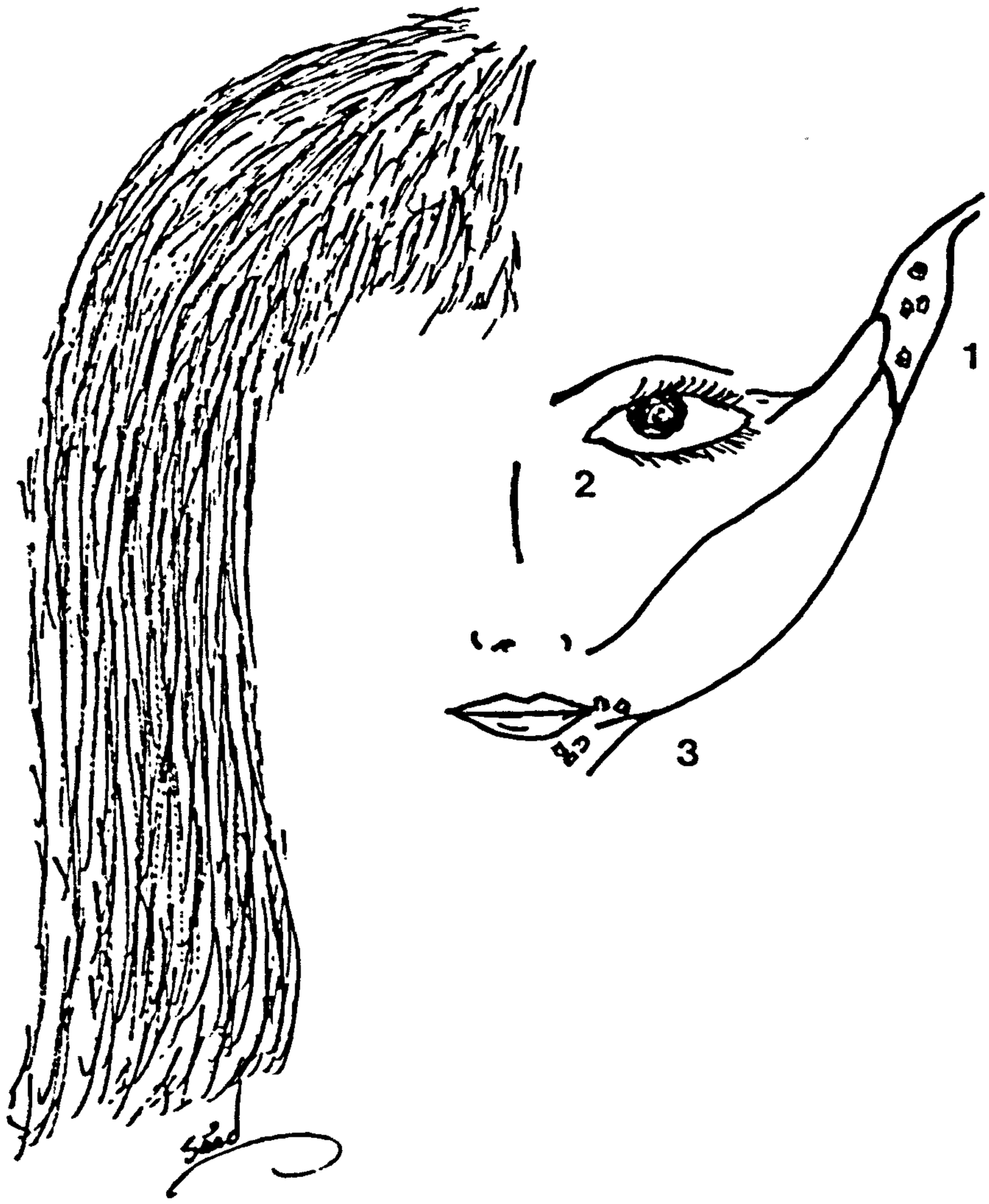
Reactivation

At any time following the establishment of latency, virus may be released spontaneously or as a result of particular stimuli so that infectious virus can be reisolated - this is called reactivation. Following reactivation, the released virus may produce observable lesions (recrudescent lesions) in the dermatome relating to the sensory ganglion, or alternatively the virus travels to the periphery, (where it probably multiplies and can be isolated) without the development of a noticeable lesion, this is called recurrence (Wildy et al., 1982). A third possible type of reactivation (in addition to the above) is that the virus reactivates in the ganglion only (Fig. 12) without subsequent shedding or lesion development (Klein, 1982). Spontaneous reactivation of HSV has been reported in the rabbit eye (Nesburn et al., 1967) and in brain (Perdrau, 1938).

Various physical and chemical methods have been used to induce reactivation. Good and Campbell (1948) were able

FIGURE 12

LEVELS AT WHICH REACTIVATION OF LATENT HSV IN HUMAN TRIGEMINAL GANGLION CAN OCCUR



1. virus reactivation in the trigeminal ganglion only (?). No virus shedding and no lesions.
2. virus reactivation in ganglion followed by shedding, e.g. in tears, no lesions.
3. virus reactivation in ganglion and multiplication of virus in the skin; recurrent herpes

to reactivate HSV in rabbit brain (with subsequent development of encephalitis) by subjecting the latently infected rabbits to anaphylactic shock (by intravenous injection of 0.2 to 0.6 cc of egg white), or by the injection of synthetic adrenaline (Schmidt and Rasmussen, 1960) intramuscularly with a total dosage of 2.0 mg. Electrical stimulation of the trigeminal ganglion has been described as inducing in vivo reactivation of HSV in rabbits, where the reactivated virus can be detected in the tear film (Green et al., 1981). Epinephrine iontophoresis to the cornea has also been used to induce reactivation (Hill et al., 1982). Attempts to induce reactivation of HSV by artificially induced fever, or by injection of cortisone (Schmidt and Rasmussen, 1960; Stevens and Cook, 1973), or adrenalin, or immunosuppressive agents (cyclophosphamide), or irradiation or implantation of tumour cells were not successful. However, intratracheal infection of the animal with pneumococci resulted in virus reactivation (Stevens and Cook, 1973). Contrary to these reports, reactivation of HSV-1 (Sekizawa et al., 1980) and HSV-2 (Kurata et al., 1978) has been successfully achieved in mice (inoculated by lip or by the corneal route) by the use of cyclophosphamide. Moreover, reactivation has been precipitated by burning with CO_2 (Openshaw et al., 1979b), hair plucking (Hurd and Robinson, 1976), U.V. and X-ray treatment (Blyth et al., 1976; Openshaw et al., 1979b; Harbour et al., 1983a) sellotape stripping (Hill et al., 1978), and application of xylene, retinoic acid, and dimethylsulphoxide (Harbour et al., 1983a). Furthermore, virus reactivation has been induced in spinal ganglia of mice after nerve sectioning (Walz et al., 1974; Price and Schmitz, 1978; McLennan and

Darby, 1980; Klein, 1982). In general, four types of stimuli have been shown to induce in vivo reactivation (Table 5); however, no common path can be recognized for the listed stimuli and it is not known whether these reactivation positive stimuli act directly on the latent virus or through a mediator, or whether they only promote the development of recurrent lesions by virus already reactivated.

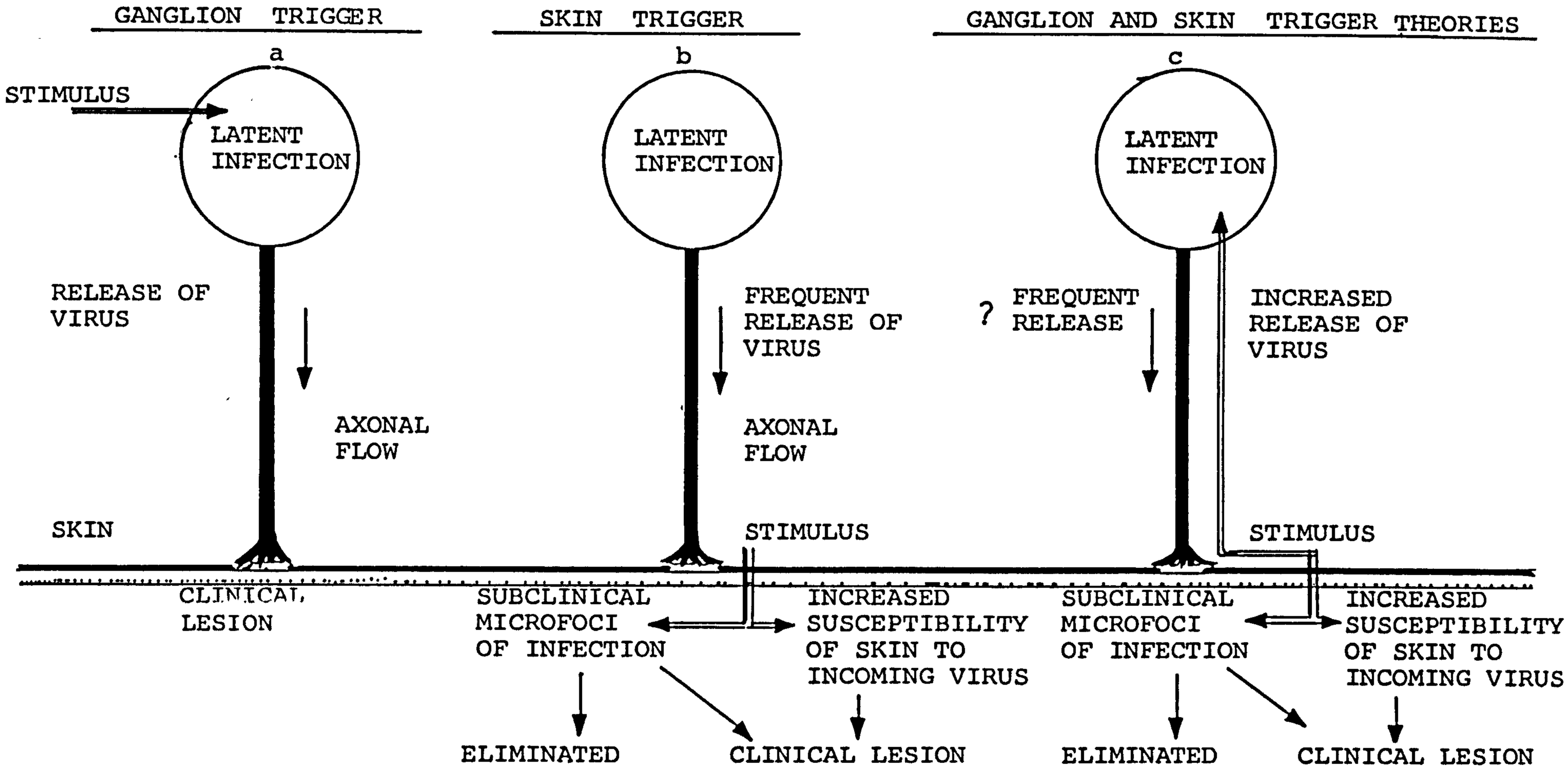
Two theories have been proposed in an attempt to explain the complexity of the reactivation process. The first theory was called the "Ganglion Trigger" theory by Hill and Blyth (1976) to distinguish it from their theory, the "Skin Trigger" theory. The ganglion trigger theory (Fig. 13a) proposed that a stimulus acts on latent infection in the ganglion to "switch on" virus from the latent state, virus then travels down to the peripheral nerve, and epidermal cells are infected so that a skin lesion develops (Cook and Stevens, 1973; Merigan, 1974; Lehner et al., 1975). The theory is widely accepted, however, it does not explain (at least in man) the development of a lesion a relatively short time after stimulation or injury, or how certain stimuli like exposure of surface skin to U.V. light can affect virus latent in the ganglion. Moreover, reactivation of virus in ganglia does not automatically produce recurrent disease (Hill and Blyth, 1976). On the other hand, the skin trigger theory (Fig. 13b) assumes that a reactivating stimulus acts on virus in the skin rather than in the ganglion. This postulates that virus is often produced in the ganglion and as a result reaches the skin cells via a nerve perhaps every few days, then microfoci of infection develop which are usually eliminated by host

TABLE 5

TYPES OF STIMULI THAT INDUCE HSV REACTIVATION IN VIVO

Stimulus	Reference
1. Manipulation of the nerve including	
a-axotomy	Price *et al.*, 1978; Walz *et al.*, 1974
b-surgical probing of ganglia	Nesburn *et al.*, 1977
2. Superinfection with pneumococcus or mucin administration	Stevens and Cook, 1973
3. Manipulation of epithelial surface	
a-arthus reaction	Anderson *et al.*, 1961
b-epilation	Hurd and Robinson, 1977
c-sellophane tape stripping	Hill *et al.*, 1978
d-local injection of prostglandin E_2 or saline	Harbour *et al.*, 1983a Blyth *et al.*, 1976
e-application of Xylene, retinoic acid and dimethyl sulphoxide (DMSO)	Harbour *et al.*, 1983a
4. Systemic treatment with immunosuppressive agents	Kurata *et al.*, 1978 Openshaw *et al.*, 1979 Underwood and Weed, 1974

FIGURE 13


GANGLION TRIGGER
SKIN TRIGGER
GANGLION AND SKIN TRIGGER THEORIES
a
b
c
STIMULUS
LATENT INFECTION
LATENT INFECTION
LATENT INFECTION
RELEASE OF VIRUS
AXONAL FLOW
FREQUENT RELEASE OF VIRUS
AXONAL FLOW
? FREQUENT RELEASE
INCREASED RELEASE OF VIRUS
SKIN
STIMULUS
STIMULUS
CLINICAL LESION
SUBCLINICAL MICROFOCI OF INFECTION
INCREASED SUSCEPTIBILITY OF SKIN TO INCOMING VIRUS
SUBCLINICAL MICROFOCI OF INFECTION
INCREASED SUSCEPTIBILITY OF SKIN TO INCOMING VIRUS
ELIMINATED
CLINICAL LESION
ELIMINATED
CLINICAL LESION

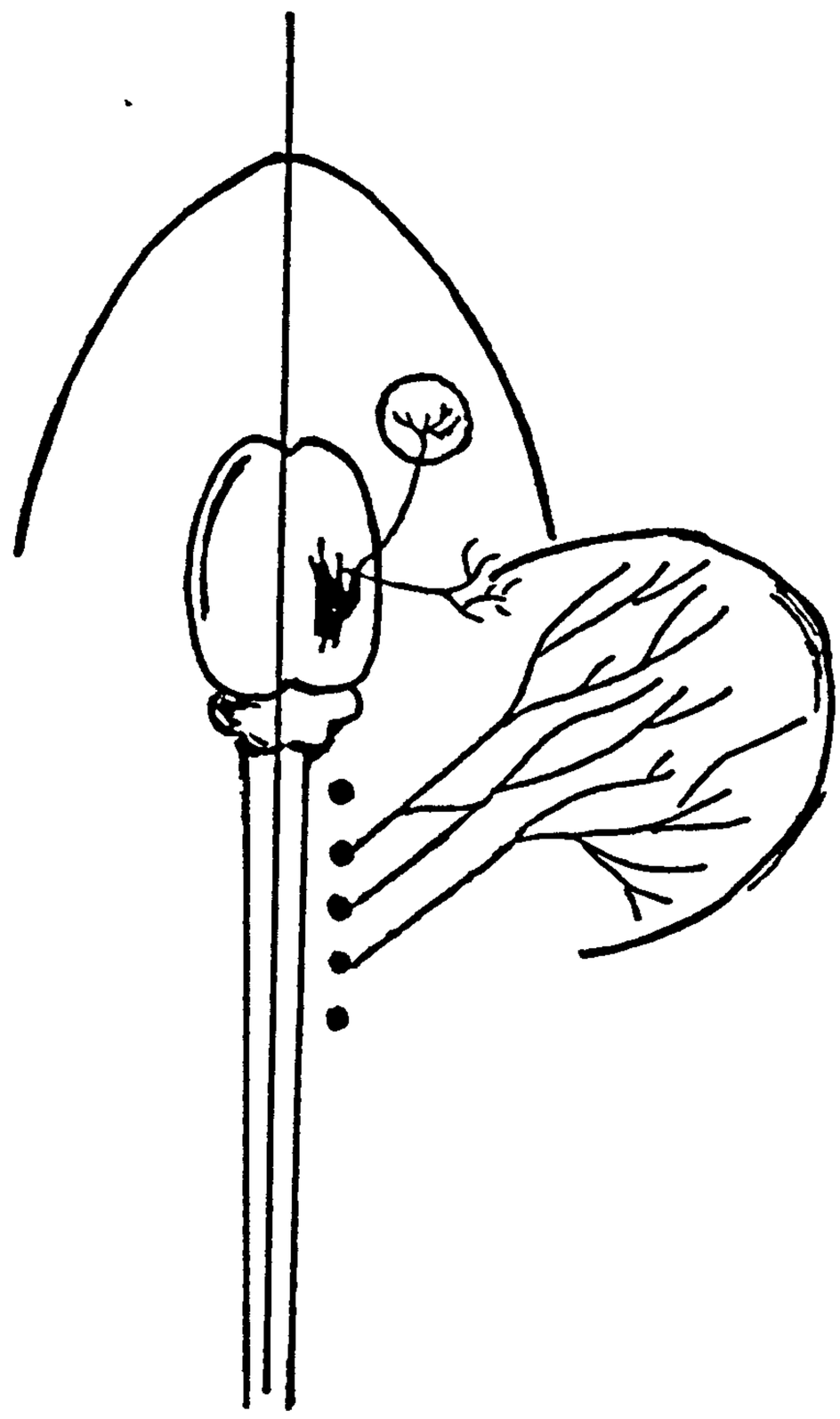
defence mechanisms; most of these infections may be abortive. Changes in the skin occasionally allow these microfoci to grow into visible lesions either by stimulating virus replication or by temporary suppression of local defence (Hill and Blyth, 1976). The theory explains that inducing agents, e.g. U.V. light and trauma do not act directly upon latent virus in the ganglion, instead the stimuli induce some physiological changes (inflammation) in the skin which provoke the development of lesions. Among the local changes that may be induced in the skin after exposure to U.V. light is the release of prostaglandin (Eaglstein and Weinstein, 1975) particularly prostaglandin E2 (PGE2). It has been shown that HSV can be reactivated by injecting PGE2 into the original site (ear skin) of infection in mice. Moreover the effect of prostaglandin was similar to the virus inducing effect of U.V. light, as the virus appeared in the skin in 20-30% of mice 2-3 days after even a single intradermal injection of PGE2 (Blyth et al., 1976). Supporting the assumption that PGE plays a major role, is the observation that PGE2 and PGF2 increase the size of HSV plaques produced in Vero cells; PGE2 does not enhance the growth of the virus by increasing the amount produced per cell, but by enabling the virus to spread from cell to cell more efficiently (Harbour et al., 1978). Furthermore, inhibitors of prostaglandin synthesis (mefenamic acid and indomethacin) decrease plaque size and inhibit the growth of virus inoculated at low multiplicity of infection. Such inhibition can be partially reversed by adding PGE2. Recently, Harbour and co-workers (1983) have shown that in the absence of serum, both PGE2 and PGF2 increase the adhesion between cells infected with HSV-1 and

uninfected cells. In addition, prostaglandin treatment did not induce polykaryocyte formation in culture infected with wild type virus. This observation supports the early suggestion (Harbour et al., 1978) that PGs facilitate cell-to-cell spread of virus. Preliminary results indicated that treatment of mice with indomethacin decreases to a great extent the number of lesions induced by xylene application (Hill, 1981). However, the skin trigger theory does not explain the mechanism of action of some inducing agents which do not act on the skin, e.g. stress, anxiety, depression, menstruation, fatigue (Klein, 1982). A further possibility is that effective inducers of recurrent disease may produce changes both in the skin and ganglia (Fig. 14c). Indeed, using the mouse ear model (Fig. 14), Hill (1981) reported that infectious virus can be found in cervical ganglia of 10% of latently infected mice 1-4 days after ear stripping with sellophane tape. Moreover, increase in uptake of ^{3}H thymidine has been noticed in cervical ganglia of normal mice, 1-2 days after ear stripping suggesting certain metabolic changes in the ganglia.

Neither the "ganglion trigger" nor the "skin trigger" theories explain how the virus can be reactivated from peripheral tissue that has no direct anatomical connection with the dorsal root ganglia (Scriba, 1977). In conclusion, it seems that recurrence must occur after reactivation and axonal transportation of the virus, and similarly recrudescence must follow recurrence, though they are not necessary consequences. Furthermore, latency and reactivation in the ganglion seem to be quite distinct phenomena from recurrence or recrudescence (Wildy et al., 1982).

FIGURE 14

MOUSE EAR MODEL



Virus injected intradermally in right ear. Latency established in 2nd, 3rd and 4th cervical ganglia. Spontaneous recurrence of clinical disease occurs at a rate of 3.5%.

Hill, 1981

Immunology of Latent HSV Infection

The relationship of the immune response to latent HSV infection is not clearly defined. Several immunological agencies have been recognized to be involved in the response to herpes infection including: macrophages (Zisman et al., 1970; Morhan and Morse, 1979), cytotoxic T cells (Pfizenmair et al., 1977; Sethi and Brandis, 1977; Nash et al., 1980b), antibody dependent cell cytotoxicity-ADCC (Shore et al., 1976), natural killer (NK) cells (Ching and Lopez, 1979; Rager-Zisman and Allison, 1980; Engler et al., 1981), delayed type hypersensitivity (Nash et al., 1980a, 1981), suppressor T and suppressor B cells (Nash and Gell, 1980; Engler et al., 1981; Nash et al., 1981), interferon (Gresser et al., 1976; Sonnenfeld and Merigan, 1979), antibody and complement (Burns et al., 1975; Oldstone and Lampert, 1979). Humoral immunity alone, has been found of limited importance both in eliminating acute infection at least in nude mice (Openshaw et al., 1979a) and in maintaining latency (Sekizawa et al., 1980). On the other hand, cell-mediated immunity (CMI) has been shown as an important factor for host protection against herpes infection (Lodmell et al., 1973; Oakes, 1975; Rager-Zisman and Allison, 1976) and in recovery from HSV-1 infection in mice (Nagafuga et al., 1979).

Impaired CMI has been demonstrated in human patients undergoing recrudescence (Wilton et al., 1972; Shillitoe et al., 1977). In support of the importance of T cells in the maintenance of latent infection is the observation provided by Kastrukoff et al. (1981). They showed that in C3H mice where CMI is acutely suppressed (i.e. mice were thymectomized one month after infecting with HSV and later

irradiated, then reconstituted with anti-Thy 1.2 hybridoma antibody plus complement treated bone marrow, and subsequently given antilymphocyte serum) a severe illness developed and the virus was observed in trigeminal ganglia, lip and CNS at a time when anti HSV antibody was present. Control (uninfected) or chronically infected but sham treated mice remained well. However, some mice in the experimental group did recover, possibly due to the action of circulating antibody.

Based on the analysis of the results in the mouse ear model (Nash and Gell, 1980; Nash et al., 1980a, b, 1981), Wildy and colleagues (1982) have proposed that three immune mechanisms are initiated following herpes virus infection: namely delayed type hypersensitivity (DTH), cytotoxic T cell responses and neutralizing antibody. It is believed (Wildy et al., 1981) that the three immune responses are active during the primary, recurrence and recrudescence stages of infection, and that T helper cells (Figure 15) play a key role essential for the induction of all three responses.

Molecular Aspects of Latent HSV Infection

Neither the virus coded functions essential for establishment and/or maintenance of latent infection, nor the mechanisms that control HSV replication in nonpermissive cells are defined. The molecular events leading to reactivation are totally unknown.

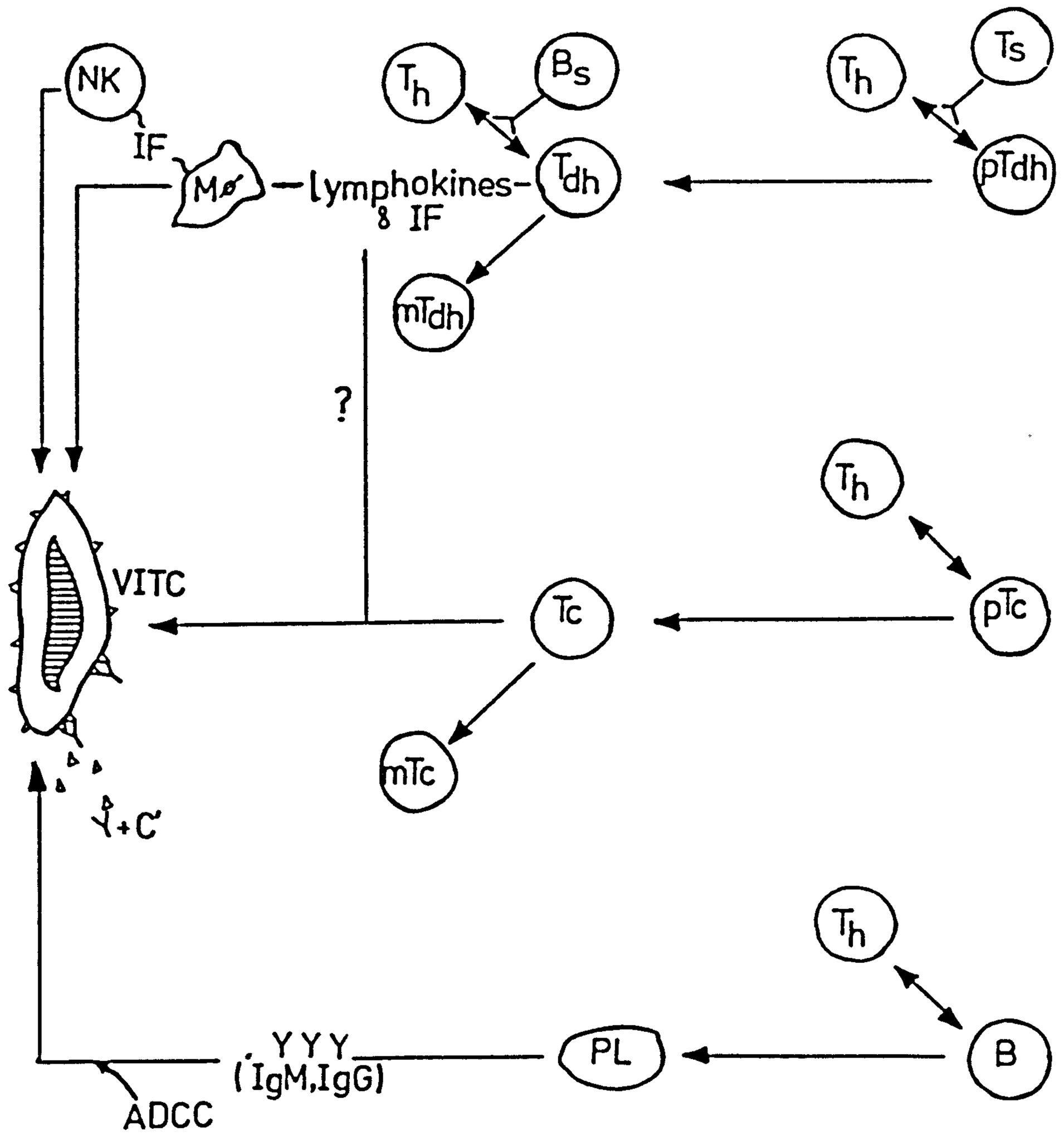
Studies with ts mutants of HSV-1 (Watson et al., 1980; Stevens, 1981; Clements and Subak-Sharpe, 1983) and of HSV-2 (Al-Saadi et al., 1983) revealed differences in the capacity of the mutants to establish latent infections in the mouse brain and ganglia. Comparison of latency phenotype (i.e. latency positive and latency negative) with the properties

FIGURE 15

DIAGRAM ILLUSTRATING THE PROPOSED T-CELL MECHANISM IN THE INDUCTION OF PROTECTIVE IMMUNE RESPONSE DEFINED BY USING THE MOUSE EAR MODEL

Ts, suppressor T cells; Th, helper T cells; Tdh, delayed hypersensitivity T cells; Tc, cytotoxic T cells; pT, precursor T cells; mT, memory T cells; Bs, suppressor B cells; B, activated B cells; pL, plasma cells; MØ, activated macrophage; NK, natural killer cell; ADCC, antibody-dependent cell cytotoxicity; IF, interferon; C', complement; VITC, virus infected cell (neurone or epidermal skin cell)

Wildy *et al*., 1982


NK
IF
Mø
lymphokines
& IF
Th
Bs
Tdh
mTdh
Th
Ts
pTdh
?
VITC
Y+C'
Th
Tc
pTc
mTc
Th
YYY
(IgM,IgG)
ADCC
PL
B

of the mutants expressed under restrictive conditions (Brown et al., 1973; Subak-Sharpe et al., 1974; Marsden et al., 1976; Stow et al., 1978; Watson and Clements, 1978; Gerdes et al., 1979) led to the following conclusions: i, viral DNA synthesis (HSV-1 and HSV-2) may be irrelevant to the establishment of latent infection, as some DNA^+ and some DNA^- mutants were equally able to establish latent infection, while other DNA^+ and other DNA^- mutants were latency negative (Watson et al., 1980; Stevens, 1981; Al-Saadi et al., 1983); ii, there is a loose correlation between absence of morphologically identifiable type 1 viral products in infected cells (neuroblastoma cells in culture; neurones in brain) and the capacity to establish latent infection; iii, HSV-genes essential to establish latent infections are not clustered on the physical and genetic maps obtained with these mutants; iv, and most important, is that one latency negative HSV-1 mutant, namely ts K, has been found to involve the gene encoding the immediate early polypeptide Vmw 175 (Preston, 1979) and since other ts mutants (ts A, ts I, ts U, ts S) which are blocked beyond the immediate early stages of the replication, are also latency negative, it is possible to conclude that at least one immediate early and one or more later functioning virus gene products are necessary for establishment and/or maintenance of the latent state (Watson et al., 1980; Stevens, 1981). Furthermore, since ts K ($latency^-$) and ts D ($latency^+$ but in the same gene as ts K) exhibit little leakiness (i.e. replication in the brain) while ts I ($latency^-$) and ts F ($latency^+$) are both rather leaky, it is concluded that there is no pattern relating latency to virus replication (leakiness) in mouse brain. It is important to

note that both ts I and ts K were found to be able to induce latent infection in mice in the dorsal root ganglia (Clements and Subak-Sharpe, 1983) which is in contrast to the previous results reported by Watson et al. (1980). However, different routes of inoculation and different mouse strains were used in each of the studies.

What determines whether cells are permissive or non permissive for HSV replication is not defined. However, it is known that replication of HSV in a permissive cell requires the utilization of cellular enzymes such as RNA polymerase II for the early transcription of viral DNA (Ben-Zeev and Becker, 1977). Other studies (Becker, 1981) revealed that treatment of permissive cells with α-amanitin (an inhibitor of RNA polymerase II) or arginine depriviation (prevention of virion assembly) leads to inhibition of the synthesis of virus progeny. Similarly the synthesis of defective interfering particles (DI) may eventually block the synthesis of infectious virus progeny. It is reported that HSV can not replicate in cell-cycle mutants that are prevented from entering the S phase of the cell cycle (Yanagi et al., 1978). Furthermore, nonstimulated T and B lymphocytes are incapable of supporting HSV replication (Kirchner et al., 1977; Westmoreland, 1978). In XC cells, (a Rous sarcoma virus transformed rat cell line) HSV expresses only 50% of its genetic information, i.e. early genes (Padgett et al., 1978) and only viral thymidine kinase (Docherty et al., 1973; Garfinkle and McAuslan, 1973, 1974) and DNA polymerase (Becker, 1981) are detectable, whereas viral DNA synthesis is prevented. However, none of these systems resemble the HSV-neurone cell interaction.

Virus and Host Factor in Latency

Viruses

It is well known that viruses differ in their virulence for certain host species. Differences in latency capacity between HSV-1 and also between HSV-2 ts mutants has been reported (Zygraich and Huygelen, 1973; Lofgren et al., 1977; Watson et al., 1980; Al-Saadi et al., 1983). Moreoever, it has been shown that HSV-2 ts mutants can be recovered from the spleen (but not from other tissues) of latently infected tree-shrew (Darai et al., 1980). It has been proposed that both highly virulent (large plaque producing) as well as less virulent (small plaque producing) viruses are able to induce latent infection (Becker, 1981). HSV-1 thymidine kinase deficient (TK^-) mutants which multiply poorly in vivo and tend to be avirulent, were found to induce latency in trigeminal ganglia of mice (Tenser and Dunstan, 1979; Field and Darby, 1980) and guinea pigs (Tenser et al., 1979) after corneal inoculation but with more difficulty (~ 10^6-10^8 pfu of input virus) than the wt viruses and their multiplication is often restricted in the nervous system. In many instances, a great difference is noticed between the wt and TK^- mutant latency capacity (Tenser and Jones, 1982). It is interesting that TK^- mutants of HSV-1 replicate efficiently in actively growing cell cultures but fail to replicate in non multiplying serum starved cells (Jamieson et al., 1974), an observation which suggests that HSV specified deoxypyrimidine kinase activity is essential for HSV replication under conditions where the host cells are not making thymidilate. It is likely that cells initially invaded in natural infections by herpesvirus will have a low level of thymidine metabolism and resemble

resting rather than non resting cells. Since neurones are non dividing cells, it is hypothesized that HSV TK expression is important in the pathogenesis of HSV infection and necessary for the establishment of sensory ganglion neurone infection (Tenser and Dunstan, 1979).

Host

The basis for susceptibility and resistance to primary or reactivated herpes virus infection has not yet been determined. In man, however, when the histocompatibility antigen types of 260 patients (who had a clear history of frequent circumoral herpes infection) were compared with 606 normal (control) subjects, a positive association between the HLA antigen A1 and the incidence of recrudescent herpes was observed (Russell and Schlaut, 1977). A suggestive association with antigen A29 and B8 was also noticed. However, this association has not been explained and it is not known whether such individuals are susceptible to infection with the virus. Nevertheless, the observation suggests that one factor that increases susceptibility to this condition in man (recrudesence) may be inherited (Russell and Schlaut, 1977).

Other experimental studies (Lopez, 1975) where 8 strains of HSV-1 were used, revealed that inbred strains of mice differ in their ability to resist infection (susceptible mice develop paralysis and death) and the mice were categorized as resistent (C57BL/6), moderately susceptible (BALB/c) or very susceptible (A/J). Other studies confirmed these differences (Kirchner et al., 1978). It has been concluded that these differences are genetically determined and appear to be mediated by natural killer cells (Lopez, 1980). Similarly intraperitoneal (IP) inoculation

of HSV-2 (Mogensen, 1976) in a series of inbred strains of mice revealed that some strains of mice were resistent while others were susceptible to focal necrotic hepatitis caused by the HSV-2. Resistance to HSV-2 seems to be sex linked (i.e. resistance gene is located on the X-chromosome) and it is involved in macrophage function (Mogensen, 1980). Spontaneous recrudescences (characterized by redness of the ear and, occasionally, vesicles) have been reported in mice, and some mice had more than one episode of recrudescence (Hill, 1975; Harbour, 1981).

In the guinea pig model described by Scriba (1975) and Donnenberg and co-workers (1980) recrudescence was much more frequent (occuring in 45% to 90% of animals) than that observed in mice, and HSV-2 was regularly isolated from extraneural sites. It is noteworthy, that the duration of clinical lesions in guinea pigs varied between 2 days and 4 weeks and recrudescence occurs on average once every 6-7 weeks (Scriba, 1975). This may explain the regular isolation of the virus described by Donnenberg et al. (1980). In addition to the mouse and guinea pigs models, recurrence was observed in rabbits following ocular inoculation (Anderson et al., 1961; Nesburn et al., 1967).

A reasonable conclusion is that both virus and host factors are involved in determination of susceptibility to HSV infection and latency.

LATENCY OF OTHER HUMAN HERPES VIRUSES

Latency of Other Human Herpesviruses

In this chapter, the latency properties of other human herpes viruses will be discussed. As far as the five human herpes viruses are concerned no reliable animal model is available to study the latency characteristics of varicella-zoster, Epstein-Barr and cytomegalo viruses, however, reported attempts for using laboratory animals in the study of such infection will be discussed.

Varicella-Zoster Virus (VZV)

Primary infection with VZV most frequently occurs early in life (childhood) and results in an acute generalized virus disease known as varicella or chicken pox. Later in individuals who have had chicken pox, VZV infection may be manifested as herpes zoster (shingles) with the development of cutaneous lesions. Evidence for the involvement of the nervous system in herpes zoster infection has been reported by Denny-Brown and Adams (1944) and earlier by Head and Campbell (1900), who had studied autopsy material from 21 cases with different forms of herpes zoster. Histopathological studies showed that the dorsal spinal ganglion is the site of extremely acute inflammatory changes that also affected the posterior, but not the anterior, nerve root. Moreover, marked secondary degenerative changes were also observed in the fibres that enter the posterior root ganglion (i.e. peripheral nerves). It has been suggested that herpes zoster of skin represents a reactivation of varicella virus which has remained latent in ganglia after an earlier episode of varicella infection (Stokes, 1959; Hope-Simpson, 1965). In fact viral isolates from patients with chicken pox and zoster were found to be antigenically (Weller and Coons, 1954) and morphologically

(Esiri and Tomlinson, 1972) identical. Moreover, their DNAs have similar molecular weights and similar restriction endonuclease pattern (Oakes et al., 1977; Richards et al., 1979). The demonstration of the presence of virus in the trigeminal nerve and ganglia during active herpes zoster infection, by immunofluorescence and electron microscopy (Esiri and Tomlinson, 1972), together with virus isolation from dorsal root ganglia (Bastian et al., 1974; Shibuta et al., 1974) strongly supports the view that VZV remains latent in the spinal ganglia after primary infection. Later and upon reactivation, the virus moves down the sensory nerve and infects the skin where herpes vesicles are produced. Unlike HSV, varicella-zoster virus has not been isolated during the quiescent period (Esiri and Tomlinson, 1972; Shibuta et al., 1974), whether this is because the virus is not there during the latent phase or because of the strong cell-associated nature of VZV (that makes the virus more difficult to recover) is not known. Although, VZV is regularly isolated from the skin lesions of herpes zoster (Esiri and Tomlinson, 1972; Bastian et al., 1974; Shibuta et al., 1974), it is not unlikely that the pain syndrome of herpes zoster can occur without subsequent rash or skin eruption (Luby et al., 1977). A condition known as "zoster-sine herpete", was described by Lewis (1958). The latter observation implies that VZV reactivation can be aborted and it is possible that reactivation is frequent but suppressed by normal host defences (Zaia, 1981). However, the role of the immune system in the reactivation of herpes zoster remains unknown. Nevertheless, some observations have been made suggesting the importance of the immune functions, for example zoster has been reported to occur in

the first year of life in certain children who have been exposed in utero to chicken pox (Hope-Simpson, 1965; David and Williams, 1979), this short latent period between the initial infection and viral reactivation (compared to the usual one in the adult) may be due to poor or immature cell mediated immunity or due to a decline in maternal antibody levels within six months of birth (Gershon et al., 1976). However, the fact that antibody to VZV does not prevent the recurrence of infection in the form of herpes zoster, suggests that other host defence mechnisms are responsible for the maintenance of virus latency. Other studies showed that humoral antibody and local interferon production (possibly mediated by sensitized lymphocytes) have an important role in preventing or restricting the dissemination of an initially local disease (Mazure et al., 1979; Stevens and Merigan, 1979). Moreover, studies of 73 patients undergoing renal transplantation, who had immunosupressive therapy, showed no relation between surgical immunusuppression (splenectomy and thymectomy), medical immunosuppressive therapy (azathioprine, prednisone and actinomycin C) or the side effects of immunsuppression (e.g. hypogammaglobulinemia and leukopenia) and the development of herpes zoster in these patients (Rifkind, 1966). In contrast following local X-irradiation (as an antirejection measure), zoster eruptions appeared primarily in the irradiated segments. These observations suggest that VZV may be latent in many sensory ganglia or in other nearby sites and can be activated by local X-rays or by some other factors like leukaemia, exposure to cold and pressure (Stokes, 1959; Rifkind, 1966).

The information about VZV infection is derived from

clinical observations as there is no suitable animal model. VZV like Epstein-Barr virus, is not pathogenic (Darai et al., 1980a) even when inoculated intravenously (IV) in juvenile or adult tree-shrews. However, Myers and co-workers (1980) reported successful infection of weanling guinea pigs with fetal guinea pig adapted VZV (FGP VZV) where nasopharyngeal virus shedding, viraemia, and specific humoral responses were noticed (in some animals); yet no evidence for virus reactivation was reported.

Epstein-Barr Virus (EBV)

Unlike herpes simplex and varicella zoster, there is no evidence for the involvement of the nerve cells as a possible site of latent infection with EBV or cytomegalovirus (CMV).

Epstein-Barr virus is the cause of Paul Bunnell positive (Henle and Henle, 1979) infectious mononucleosis (IM), however, the virus is also been associated with two other diseases of man, namely Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC) reviewed by Miller (1978). Primary infection with EB virus usually occurs in early childhood (Henle et al., 1968; Kafuko et al., 1972). The infection in the infant remains essentially silent (Biggar et al., 1978) and once infected a person becomes a life long carrier, shedding virus periodically (Smith, 1978). Reactivation of EBV is less well documented partly due to the difficulty in viral detection, and moreover it appears that EBV causes no symptoms upon reactivation (Strauch et al., 1974; Naraqi et al., 1977). In one of the studies (Strauch et al., 1974), human leucocyte immortalization activity (an assay of EBV biological activity) was detected in throat washings of three out of twenty (15%) healthy,

renal unit associated personnel, and in 18% of general hospital outpatients (Chang et al., 1973) suggesting that asymptomatic excretion of EBV is common among the general population. It has been proposed (Pagano and Shaw, 1979) that infection with EBV involves primary (epithelial) and secondary (B-lymphocyte) cell types. The oropharyngeal epithelium is thought to be the site for vegetative virus replication (during the course of infectious mononucleosis) from which the virus is subsequently excreted (Lemon et al., 1977), presumably reaching the tonsils where infection and transformation of B-lymphocytes occurs. The above finding is inconsistent with early observations that virus shedding can occasionally be localised in the anterior oropharynx (where lymphoid tissue is scant but epithelium is abundant) rather than the posterior pharynx and tonsillar areas (Neiderman et al., 1976). Unlike the epithelial cells, EBV replication in lymphocytes is repressed and a state of latent infection induced (Pagano, 1975).

Explantation of EBV latently infected lymphocytes results in virus replication and cellular proliferation reviewed by Pagano and Nedrud (1981). Occasionally, however, latent EBV genomes can persist in epithelial cells. Evidence for the latter fact has been reported by Wolf and colleagues (1973) who, by using in situ hybridization, demonstrated that the EB viral genome is harboured by epithelial cells of nasopharyngeal carcinoma. Similarly, Huang and co-workers (1974) reported the presence of EBV associated nuclear antigen (EBNA) in nasopharyngeal carcinoma cells. Furthermore, EBV has been recovered (Morgan et al., 1979) from cell free material in saliva or in secretions from parotid-gland orifices or ducts (by

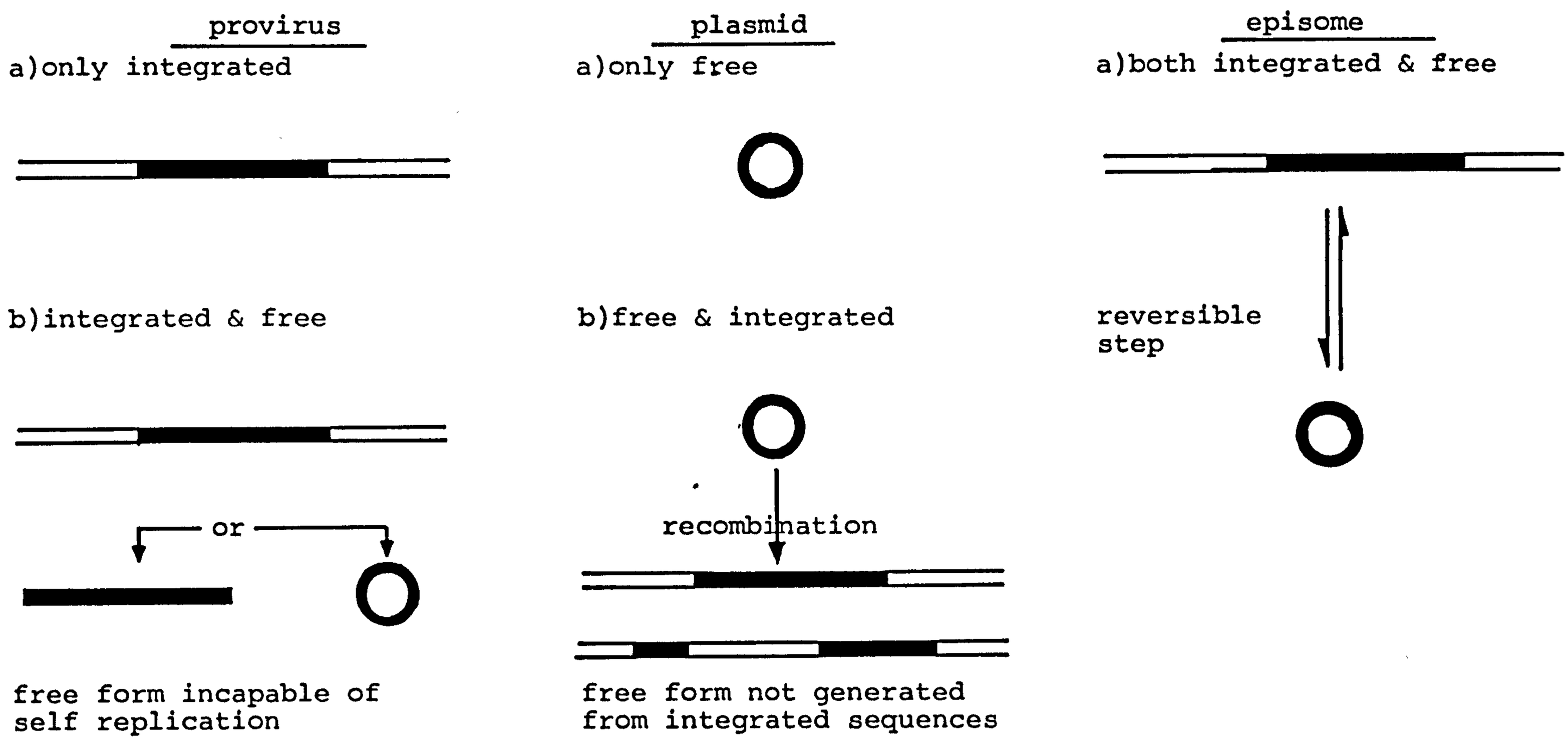
cannulation of Stensen's duct).

While the state of the HSV genome in latently infected cells is not yet understood, little is known about the state of EBV genome during latency. The virus DNA has been characterized as a linear molecule with a molecular weight ~ 10^8 (Adams and Lindahl, 1975) and it has been found that in a nonproducer latently infected cell line (Raji cell line which was derived from a Burkitt's lymphoma) part of EBV DNA is present as an integrated form, while the majority of the DNA occurs in free form in the cell nuclei (Nonoyama and Pagano, 1972; Tanaka and Nonoyama, 1974). This free form of viral DNA, described as EBV "plasmid" or "episome" (Nonoyama and Pagano, 1972; Lindahl et al., 1976) exists as a supercoiled molecule within latently infected cells (Adams and Lindahl, 1975; Adams, 1979; Shaw et al., 1979). Further, EBV episome replication is associated with cellular chromosomal (S-phase) replication (Hampar 1974) and complete copies of the virus genome persist in replicating cells (Epstein and Achong, 1977). So it appears that both integrated and non integrated (Fig. 16) viral DNA occurs in the transformed cell lines (Lindahl, 1976; Anderson and Lindahl, 1978).

Activation of EBV DNA in non productive cell lines by the use of different inducing agents (phorbol ester - TPA, sodium butyrate, corticosteroids, antihuman IgM and low temperature) were generally unsuccessful (Hampar, 1979). However, in addition to the limited spontaneous virus production in the virus-producing P3HR-1 cell line (Hinuma et al., 1967) the induction of the latently infected cells to express viral antigens and produce virus (within such productive cell lines) was greatly been enhanced by using

FIGURE 16

MODELS FOR THE MAINTENANCE OF LATENT EBV GENOMES IN TRANSFORMED CELLS



Double-stranded EBV DNA molecules are depicted graphically(solid black areas)in various associations with cellular DNA(clear areas).

these inducing agents (Hampar, 1979). Moreover, when non-producer (Raji cells) were treated with iododeoxyuridine (IUdR) only the early antigen (EA) component was detected (no significant increase in EBV DNA and no virus particles were observed), while similar treatment of somatic cell hybrids, produced by fusion of Hela and Raji (D98/Raji), resulted in synthesis of early antigen, EBV DNA and virus particles (Glaser and Nonoyama, 1974). On the other hand, the somatic cell hybrid (D98/HR-1), produced by fusion of HR-1 cells (producer) to human Hela cell variant D98, was shown to contain EBV DNA but the genome was repressed (i.e. no detectable specific virus antigens were synthesized). However, when the hybrid was treated with IUdR or BUdR, synthesis of EBV DNA, EA, virus capsid antigen (VCA) and virus particles were observed (Glaser and Rapp, 1972; Glaser et al., 1973; Klein et al., 1976). These facts suggest that the degree of induction of latent virus (in producer and non producer cell) is under the cellular control rather than due to differences in EBV genomes "episomes" present in these two cell lines.

Cytomegalovirus (CMV)

Cytomegaloviruses are a group of viruses which in addition to human cytomegalovirus (HCMV), includes species specific viruses of other animals like the murine CMV, simian CMV and guinea pig CMV. Though there is structural and biological similarity, there is lack of genetic relatedness between these viruses as determined by nucleic acid hybridization and restriction endonuclease analysis (Huang and Pagano, 1974). Primary HCMV infection is most likely to be asymptomatic and the infection can be acquired perinatally (with clinical manifestations ranging from

silent infection to severe cytomegalic inclusion disease and intrauterine death), or during the childhood or adulthood period (Weller, 1971a, b). And it appears that CMV is endemic in all human societies. The virus, in addition to being non-specifically associated with a variety of clinical syndromes, is now recognized as the cause of certain clinical disorders (fever, leukopenia, thrombocytopenia, a mononucleosis-like illness, pneumonitis and hepatitis) early after renal transplantation, and asymptomatic virus excretion (in saliva and urine) continued up to 2-14 years postrenal transplantation (Cheesman et al., 1979). CMV has been isolated from saliva, urine, blood and human milk (Hayes et al., 1972) and faeces (Cox and Hughes, 1974). Asymptomatic excretion in the throat and in urine is common particularly in congenitally infected infants (Reynolds et al., 1974).

The clinical and epidemiological observations strongly support the assumption that after primary infection HCMV can establish latent infection with subsequent recurrence or reactivation upon disturbance of virus host relationship (Weller, 1971b). Experimental studies on the site and mechanism of virus persistence and reactivation in man is not possible. Nevertheless, CMV "inclusions" (characteristic cytomegalic cells) have been located in epithelial cells of renal tubules (Fetterman et al., 1968) and in salivary glands (Huang and Pagano, 1977). Further, Diosi and co-workers (1969) have reported the isolation of CMV from explanted leucocytes of healthy blood donors. The demonstration of CMV in human semen (Lang et al., 1974) raises the possible persistence of latent viral genomes in spermatozoa.

Since cytomegaloviruses are highly species specific, studies of HCMV in laboratory animals has been limited. However, acute and chronic CMV infection of mice with mouse salivary gland cytomegalovirus (MCMV) is very similar to that in man (Brodsky and Rowe, 1958; Medearis, 1964). Thus a murine model of CMV infection may provide important and relevant information about the disease in man.

Olding and colleagues (1975) could reactivate and reisolate MCMV from B-lymphocytes of latently infected mice (mice were infected in utero or at birth) but only upon co-cultivation of the explanted cells with histocompatable (allogenic) mouse embryo cells (MEC) or when B-lymphocytes were stimulated by adding lipopolysaccharide (LPS) to the culture. Other studies failed to detect virus in brain, thymus, liver, kidney, urine or serum of latently infected mice by co-cultivation techniques or by host DNA-MCMV DNA hybridisation. Virus could be activated from spleen cells (Olding et al., 1976), and MCMV DNA was detected at levels equivalent to 3-4 virus genomes/100 spleen cells and 2 virus genomes/100 salivary gland cells. Further studies showed that MCMV was harboured in the reproductive tissues (sperm cells) of both latently and acutely infected male mice (Dutko and Oldstone, 1979) and in macrophages (Brautigam et al., 1979). In addition, Cheung and Lang (1977) have described a mouse model that mimics many of the features of HCMV infections which are associated with transfusion and perfusion. Not only transfusions from uninfected donors (mice) into latently infected mice activated MCMV in heterologous and homologous recipients, but also infection can be transmitted to healthy mice by the inoculation of blood from previously inoculated animals who were apparently

virus-negative. Reactivation and dissemination of the latent MCMV following the use of immunosuppressive therapy (antilymphocyte serum and cortisone) has also been reported (Jordan et al., 1977; Shanley et al., 1979).

The state of the latent virus genome within the cell is not known, but in vitro study on the analysis of human cytomegalovirus nucleoprotein complexes in acutely infected human embryo lung cells indicated that virus DNA is present in a nucleosome-like structure (Jeor et al., 1982). Recent studies on the association between HCMV DNA and cellular DNA in latently infected human embryonic fibroblast cells indicate the integration of viral DNA (Gadler and Wahren, 1983). Furthermore, since viral replication was initiated after the removal of phosphonoformic acid (PFA), it is assumed that the complete viral genome persists in cells. However, it is not known whether the genome persists as a fragmented or as a continuous sequence, and the situation in vivo has not been determined.

MATERIALS AND METHODS

MATERIALS

Virus

The HSV-2 wild type virus used in this study was strain HG52; from which all the type 2 temperature-sensitive (ts) mutants used in this study, had been derived by 5-bromodeoxyuridine mutagenisation (Timbury, 1971; Halliburton and Timbury, 1973, 1976). The studied mutants were ts 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13.

Tissue Culture Cells

Both baby hamster kidney cells (BHK21/C_{13}) described by Macpherson and Stoker (1962) and human embryo lung cells (HeLu) were used.

Radiochemicals

^{32}P orthophosphate (carrier-free) was purchased from the Radiochemical Centre, "Amersham".

Chemicals For Liquid Scintillation Counting

(2, 5 diphenol-oxazole) PPO was obtained from Koch-Light Laboratories Ltd. The toluene used was Analar Grade (B.D.H. Ltd). Scintillation fluid contained 5 g PPO per litre of toluene, 5 ml of the solution was added to screwtop plastic scintillation vials.

Tissue Culture Media

BHK21/C_{13} cells were grown in burlers, using Glasgow modified Eagles medium (Busby et al., 1964). The medium was supplemented with appropriate antibiotic/antimycotic solutions (100 units/ml of penicillin, 100 ug/ml Streptomycin, 0.02 ug/ml of antimycotic agent, N-butyl-p-hydroxybenzoate). In some cases, the antibiotic/antimycotic concentration was increased to (150 units/ml penicillin, 150 ug/ml Streptomycin and

0.04 ug/ml of antimycotic), Gentamicin was also added at the concentration of 25 ug/ml.

The medium was also supplemented with tryptose phosphate broth and different concentrations of either normal or heat inactivated calf serum (CS) or fetal calf serum (FCS).

Eagles Supplemented Medium

5% (v/w) foetal calf serum .....
(EFC5)

5% (v/v) pooled human serum .....
(EHu 5%)

10% (v/v) glycerol + ETC10 .....
(ETC10gL)

10% (v/v) tryptose phosphate broth + 10% (v/v) calf serum
(ETC10)

10% (v/v) calf serum .....
(EC10)

50% (v/v) foetal calf serum .....
(EFC50)

G.M.E.M.- P.I.C. This is Glasgow modified Eagles Medium, phosphate free, supplemented with 1% calf serum

Medium For Culturing Dissociated Ganglia

Cells from dissociated dorsal root ganglia (DRG) were grown in CMRL - 1066 (Gibco) medium supplemented with 2 mM glutamine, 6 g/L glucose, 25 mM potassium chloride KCl, 25 ug/ml gentamicin and 50% (v/v) heat inactivated fetal calf serum.

Standard Solutions

1. Phosphate buffer saline (PBS).

A solution of 0.17 M NaCl, 0.0034 M KCl, 0.001 M NaH_2PO_4, 0.002 M KH_2PO_4 in distilled water pH 7.2 (Dulbecco

and Vogt, 1954)

2. PBSCS

The PBS supplemented with 10% (v/v) calf serum.

3. PBSA + gentamicin.

A phosphate buffer saline solution "A" (i.e without B+C constituents) supplemented with 25 ug/ml gentamicin.

4. Versene (EDTA).

Ethylene diaminetetraacetic acid (0.006 M) dissolved in PBS containing 0.002% (w/v) phenol red.

5. Trypsine-Versene.

One volume of 0.25% (w/v) Difco trypsin (dissolved in tris saline) and four volumes of 0.006 M versene.

6. Formal Saline.

A solution of 4% (w/v) formaldehyde in 0.085 M NaCl, 0.1 M Na_2SO_4.

7. Standard Saline Citrate (SSC).

A solution of 0.15 M NaCl, 0.015 M in sodium citrate in distilled water.

8. 1 X E Buffer

TRIS	43.6 g/lit
NaH_2PO_4 H_2O	46.8 g/lit
Na EDTA	3.7 g/lit
H_2O	1 lit

9. SDS in SSC.

A 5% solution of sodium dodecyl sulphate (SDS) in SSC, pH 7.0.

10. Giemsa Stain. A 1.5% (v/v) suspension of giemsa stain in glycerol heated to 56° for 120 minutes, diluted with an equal volume of methanol and filtered.

11. Saturated Phenol Solution

Phenol solution saturated with 75 mmol/l NaCl,

50 mmol/l EDTA, (pH 8.0).

12. RNA-ase mixture.

The mixture was prepared freshly each time and boiled for 10 minutes immediately before use.

(1 ml distilled H_2O + 100 ug RNA-ase A + 250 units of RNA-ase T_1)

Restriction Endonuclease Enzymes

These were purchased from Bethesda Research Laboratories (BRL). The enzymes were EcoRI, Bam HI, Bgl II, Hind III and Kpn I.

Reaction Mixtures

1. Eco Reaction Mixture.

Used with EcoRI endonuclease enzyme.

10 ml 1M TRIS pH 7.5

10 ml 1M NaCl

500 ul 1M $MgCl_2$

2. Standard Reaction Mixture (R. Mixture).

Used with endonuclease enzymes Hind III, Bgl II and Bam HI.

4.2 ml 1M TRIS pH 7.5

4.2 ml 1M $MgCl_2$

4.0 ml 1M KCl

87.6 ml deionized H_2O

3. Low Salt Reaction Mixture.

Used with Kpn I endonuclease enzyme.

4.2 ml 1M TRIS pH 7.5

4.2 ml 1M NaCl

4.2 ml 1M $MgCl_2$

87.4 ml deionised H_2O

Each of the above mixtures were aliquoted in 10 ml aliquots, autoclaved, then 30-35 ul of (v/v)

β-mercaptoethanol added to each 10 ml volume before they were cooled down and stored at -20°C.

4. Restriction Endonuclease Stop Mixture (R.E.)

The mixture used to stop restriction endonuclease reactions was:-

2 g Ficoll

0.54 g EDTA

10 ml of 10 x E (buffer)

10 ml of H_2O

Bromophenol blue (BPB) is added to produce a desirable colour.

Collagenase

A 0.25% (w/v) of collagenase (Worthington) was prepared in PBSA and filtered.

Anti HSV Serum

Poly-specific anti serum directed against HSV was used. This was purchased from Wellcome Laboratories.

Cells Specific Marker

The neurone specific marker (monoclonal antibody) designated A_2B_5 (Eisenbarth et al., 1979) was used at concentration of 1/40 (v/v) in PBSA.

Laboratory Animals

Five different strains of mice were used; these were A, PIRBRIGHT, C57BLACK/6-J, BALB/C and BIOZZI. The mice were bred on site at the breeding unit of the Institute of Virology.

Miscellaneous Material

Plastic petri dishes, microtitreplates, plastic tissue culture bottles, Linbro wells were purchased from Flow Laboratories, Irvine, Scotland.

Kodirex X-ray films were supplied by Kodak Ltd, London.

METHODS

Management of Cell Stocks

Stocks of BHK21/C_{13} and HeLu cells were prepared for storage in liquid nitrogen (-170°C) as follows.

A cell suspension at a concentration of $2x10^8$ cells/ml was prepared in ETC10-gl. The cell suspension was dispensed as 1 ml aliquots in screwcap vials and gradually brought down to liquid nitrogen temperature.

Cells were checked for any bacterial contamination by plating a sample on two Blood agar and on two nutrient agar plates, which were then incubated at 31° and 38°C and checked for growth of contaminants. Cells were also routinely checked for the presence of mycoplasma contaminants. (Chen, 1977).

Tissue Culture

(1) Burler Seeding

BHK21/C_{13} cells were seeded (at a density of $2x10^7$ cells/bottle) with 200 ml ETC10 in 80 oz burlers (Winchester bottles) in an atmosphere of 5% CO_2 and then incubated in the hot room (37°C). Three days later, when a confluent monolayer had been formed, some of the burlers were used for virus stock production, while the cells of other burlers were harvested, by two washes in trypsin-versene and resuspensed in ETC10 at a density of 10^6 cells/ml. The cells were then used to reseed further burlers for subsequent experiments. Cell suspensions were stored at 4°C for up to 5 days.

(2) Linbro Well Seeding

Linbro wells (16 mm well diameter) were seeded with BHK21/C_{13} at a density of $2x10^4$ cells/well in 0.5 ml ETC10

and incubated at 37°C in 5% (v/v) CO_2 in humidified incubator. When the cells were semiconfluent (24 hour later), they were used to assay supernatant fluids from footpad tissue cultures.

(3) Seeding BHK21/C_{13} in Microtitre Plates

Cells were seeded in flat bottomed multi-well tissue culture microtitre plates at a density of $6x10^3$ cells/well in 0.15 of ETC10. The plates were then incubated at 37°C in a humidified incubator containing 5% (v/v) CO_2. When the cells were semiconfluent (24 hours later) the explanted ganglia supernatants were replaced and assayed (2-3 times a week) by adding them to individual wells of microtitre plates.

Production of Virus Stock

The growth medium ETC10 was removed from confluent monolayers of C_{13} cells in burlers and the cells infected with virus at a multiplicity of one plaque forming unit (pfu)/300 cells in 25 ml ETC10.

The infected culture was then incubated at 31°C for at least three days or until extensive cytopathic effect (CPE) was obtained (80% or more of the cells being destroyed). These cells were then harvested by scraping them from the glass (using sterile glass beads if necessary), centrifuged at 500xg for 10 minutes at 4°C and resuspended in 5 ml of the supernatant fluid. The suspension was then sonicated for 2-3 minutes to release cell associated virus. After a further centrifugation at 500xg for 10 minutes, the supernatant was then poured off and kept. The sonication procedure was repeated and the supernatants pooled. The virus stocks were then dispensed in 1 ml aliquots and stored at -70°C.

Sterility Test

This was carried out for every virus stock preparation or isolate. A loopful of the virus suspension was streaked on two Blood Agar and two Nutrient Agar plates. A plate of blood and of nutrient agar were incubated at 37°C and the other pair incubated at 31°C. The plates were checked daily for any bacterial growth.

Only sterile HSV stocks were retained.

Titration of Virus Stocks

Virus was assayed using confluent monolayers of BHK21/C_{13} cells seeded onto 40 mm tissue culture petri dishes at a density of 10^5 cells/petri dish in two ml of ETC10. Virus was serially diluted (10 fold dilution steps) in PBSCS and plated onto confluent BHK monolayers on 40 mm petri dishes (0.1 ml/petri dish) from which the medium had been removed. The virus was allowed to absorb for 45-60 minutes at 38°C, then the monolayers were overlaid with 2 ml of EHu 5%.

Duplicate petri dishes were used for each dilution at each temperature (31° and 38°C) and incubation continued for 3 days at 31°C and 2 days at 38°C.

After staining with giemsa, plaques were counted using a Wild (M7A) binocular dissecting microscope.

Inoculation of Mice for the Establishment of Latency

Footpad inoculation

Both male and female mice were injected at weaning (3-4 weeks) with the appropriate dose of virus (in 0.025 ml), into the right rear footpad using a 26 G 3/8 needle. B.D (Cat. No. US110 11). For the inoculation the virus was diluted with PBSCS 10. The remaining virus suspension was also titrated on C_{13} cells at 31°C and 38°C after each

inoculation.

Mice were examined daily for the first three weeks after inoculation for the presence of any observable lesion. At least three months, after footpad inoculation and in the absence of clinical lesions, the survivors were killed with chloroform and tissue explantation carried out.

Intracerebral inoculation

For intracerebral inoculations, the virus suspension was similarly diluted with PBSC10 to the required concentration and 0.05 ml was inoculated intracerebrally while mice were under ether anaesthesia.

Explantation and Cultivation of Tissues from Latently Infected Mice

Mainly two types of tissue were chosen for explantation from latently infected mice, the 'footpad' (FP) and the 'Dorsal Root Ganglia' (DRG). In some experiments in addition spinal cord and brain were also explanted.

The explantation procedure was as follows:

(1) Footpad Tissue (FP). Both hind feet (the inoculated and uninoculated) were cleaned by washing with absolute alcohol, then a sliver of footpad tissue was excised with separate sterile instruments from both sides and dipped separately in 1 ml of absolute alcohol for about 20 seconds.

The explanted tissue was then separately washed twice in 1 ml of EFC50, then each fragment was placed separately in a microtitre well, epidermis facing upward. EFC50 was added, sufficient just to cover the surface of the explanted tissue and incubation at 31°C commenced.

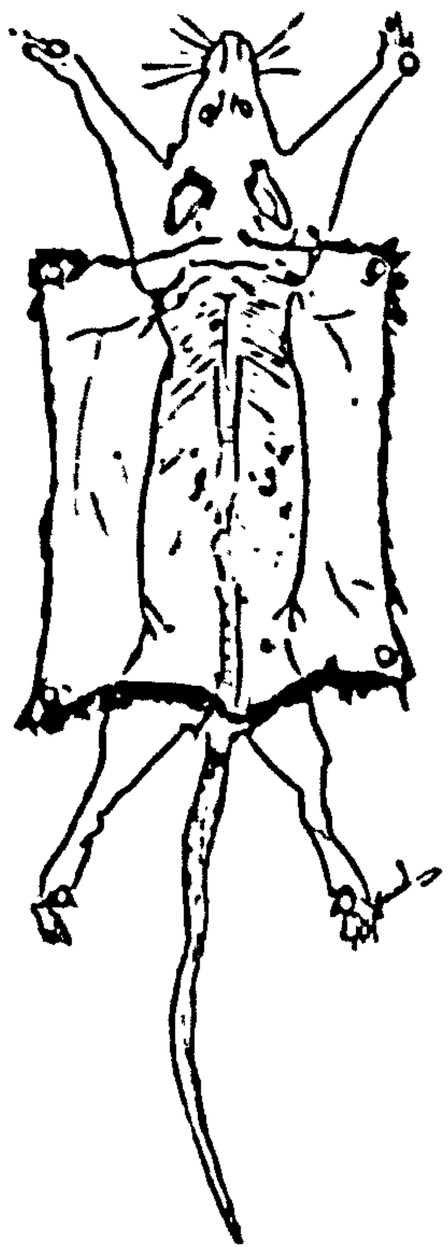
(2) Dorsal Root Ganglia (DRG). Figure (17) shows in sequence, the stages of mouse dissection and explantation of the DRG.

FIGURE 17

DIAGRAMMATIC REPRESENTATION OF THE METHOD FOR DISSECTION AND EXPLANTATION OF THE MOUSE DORSAL ROOT GANGLIA

A mouse fixed on dissecting board in a dorso-ventral position. The back of the mouse was carefully washed with absolute alcohol. The skin was dissected away from the body (on either side) and fixed (A); longitudinal incisions were made laterally to the vertebral column so as to separate the column from the supporting muscle. A cut was made through the vertebral column (transversely) at the level of the last two ribs (B); the dorsal lamina of the vertebral column was separated and removed (C); the spinal cord was then removed and the dorsal root ganglia (DRG) identified under the dissecting microscope (D).

A



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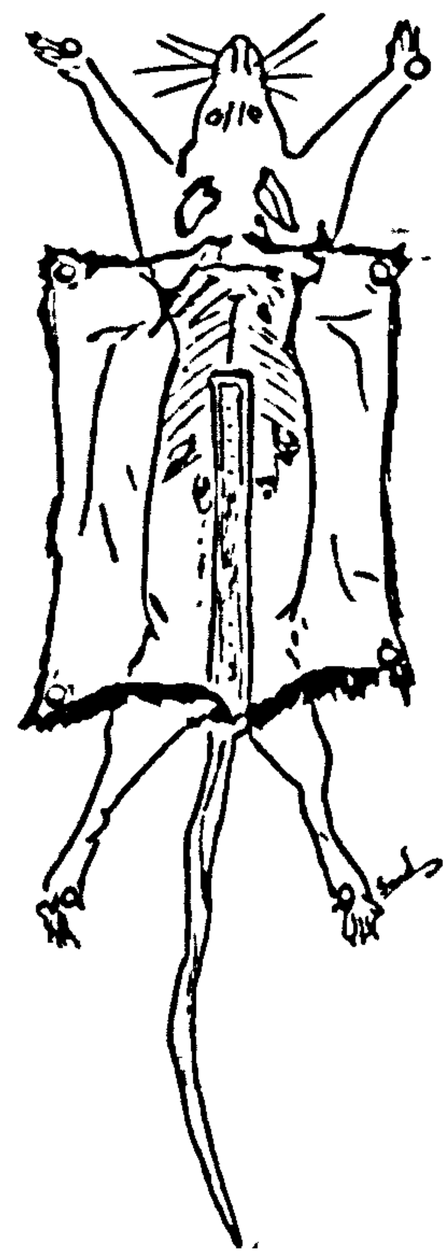

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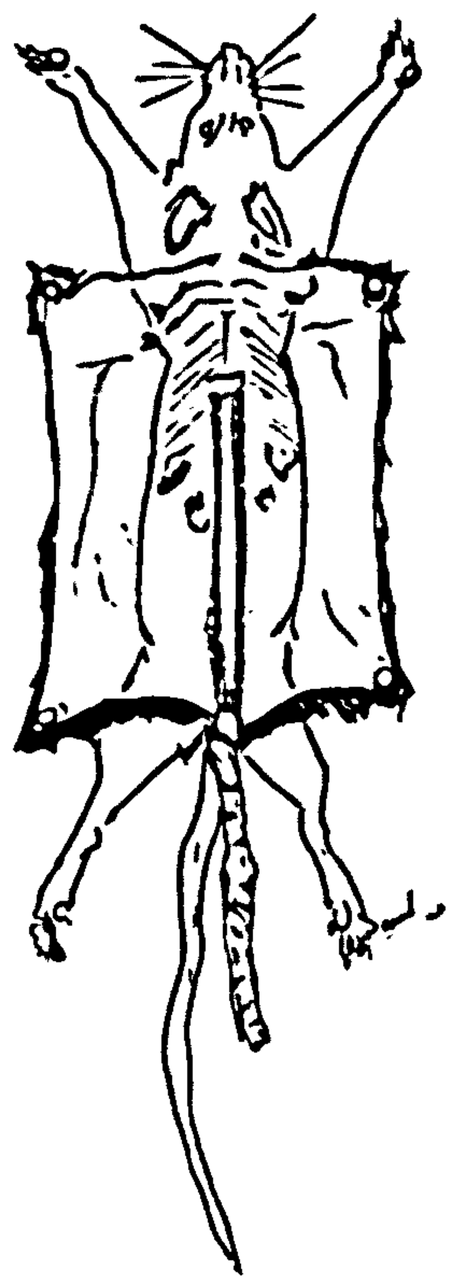

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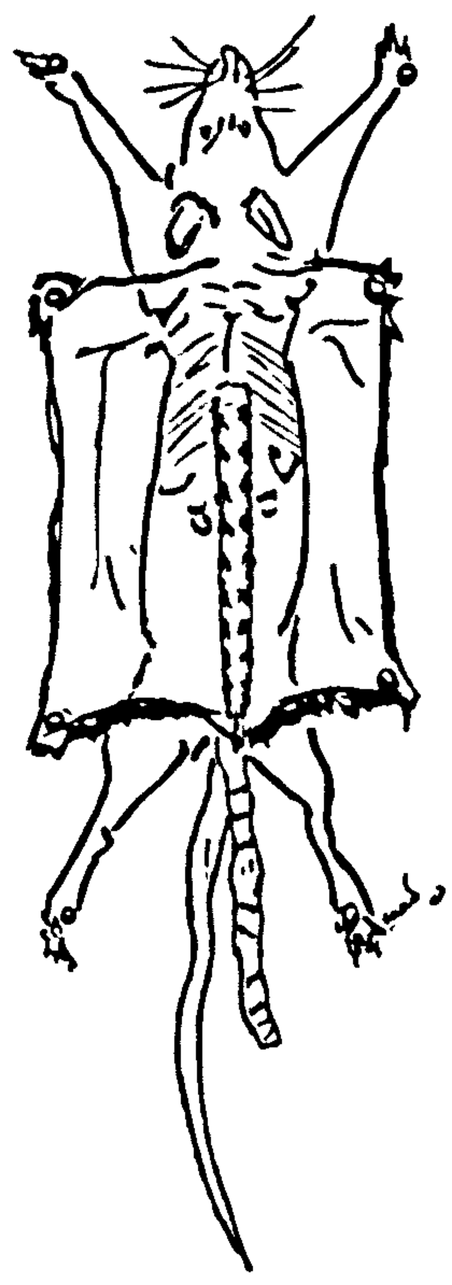


Explanted ganglia were either incubated individually as organ cultures or dissociated then incubated as cell cultures.

(A) DRG - Organ culture

Nine dorsal root ganglia were explanted from the right side comprising the last two thoracic, all six lumbar and the first sacral ganglia. Two ganglia (from the lumbar region) were also explanted from the left side as an internal control.

The explanted DRG were washed separately with 150 ul of prewarmed PBSA.G, then placed into separate wells of a microtitre plate containing 150 ul of EFC50/well. Microtitre plates were incubated at 31°C in a humidified incubator under an atmosphere of 5% CO_2 in air.

(B) Dissociation of DRG

The last two thoracic, all lumbar and first sacral DRG from each side (left, right) were removed aseptically and washed, as above, in prewarmed PBSA.G. The ganglia from 4-5 mice were pooled for each experiment. Throughout all procedures, the ganglia from the left, right or uninfected controls were treated separately. Dissociated cell cultures of adult mouse DRG were prepared by gently teasing the pooled ganglia with fine sterile forceps and then transferring to vials containing 2 ml of 0.25% collagenase (Worthington) in which they were incubated for 2-3 hours at 37°C. Vials were shaken gently at 15 minute intervals. The ganglia were then washed twice in PBSA.G and dissociated by trituration in CMRL-1066 medium through 200 ul size Eppendorf plastic pipette

tips. The resulting cell suspension was then passed through sterile nylon mesh, centrifuged at 500xg for 10 minutes, and the cell pellet resuspended in 0.5 ml of CMRL-1066 medium. 0.1 ml of cell suspension was plated onto 13 mm collagen-coated glass coverslips ($5x10^5$ cells per coverslip) in Linbro Multiwell plates and incubated at 37°C. After 24 hours incubation, another 0.4 ml of CMRL-1066 medium was added to each coverslip and reincubated. Cultures were screened daily for the presence of released HSV by plating 0.1 ml of culture supernatant onto BHK21/C_{13} cells and observed for cpe.

(3) Brain and Spinal Cord Tissues from Latently Infected Mice.

Either one cm^3 "arrowed needle plug" or the whole brains of latently infected mice were removed aseptically, then washed with PBS + gentamicin. Tissues were then cut into small, thin pieces and cocultivated with HeLu cells in 40 mm petri dishes in EFC 5% (each single piece is allocated one petri dish). Cultures were then incubated at 31°C and checked daily for CPE. Cultures were kept as long as possible (usually up to 4 weeks). After that period of incubation, cells and brain pieces from negative cultures, were harvested, sonicated and replated to a suitable number of C_{13} petri dishes. Spinal cord was removed, washed then gently homogenised in 5 ml of EFC 5% either by passing through sterile hypodermic syringes of different gauges, G19 - G26 (B.D) or slicing into small pieces. Spinal cord suspension or pieces were then cocultivated with HeLu cells in petri dishes (0.5 ml to each petri dish) or (one piece/each petri dish). Cultures were then incubated at 31oC and observed for as long as possible (3-4 weeks).

Negative cultures were harvested either by sonication and/or freezing and thawing cycles, then replated on BHK cells.

Mouse Inoculation for the Study of Virus Replication During the Acute Stage of Infection

The intracerebral route of inoculation was used to inoculate either HSV-2 wt or one of the ts mutants. Procedure was as for the establishment of latency in the mouse brain, except that mice were killed after a shorter period of time following inoculation. The whole brains from acutely infected mice were removed at 5', 10', 15', 30'; 1, 2, 6, 12 hours, 1, 2, 3, 4, 5, 6, 7 and 12 days after inoculation. At least two mice were used for each particular time point choosen in this study.

After removal, brains were individually allocated (without washing) into Universals containing 5 ml of EC10. Brains were then harvested by homogenisation, sonication followed by freezing and thawing cycle.

Brain suspensions were centrifuged at 4000 rpm (in MSE cold spin) for 30' at +4°C, and the supernatant collected as S1. Pellets were resuspended in 5 ml of fresh EC10, sonicated, then centrifuged as before. Supernatant was collected, pooled then titrated at both 31°C and 38°C by plating 0.1 ml on C_{13} seeded petri dishes. Three days later petri dishes were fixed and stained and the plaque titre determined.

Immunofluorescence experiments

Indirect immunofluorescence experiments were performed to ascertain the identity of the neural cell types supporting HSV reactivation in cultures from dissociated DRG.

The monoclonal antibody A_2B_5 (neurone specific marker)

and poly-specific antiserum directed against HSV (rabbit anti-HSV) were used in these experiments.

Cells from dissociated DRG were grown on collagen-coated coverslips at 37°C in linbro-wells. After a time interval either when a CPE was first noticed in cultures of the dissociated DRG or when there was virus release in the supernatant (indicated by plating the supernatant on BHK21/C_{13} cells), the coverslips were picked up, washed and individually treated with 40 ul of A_2B_5 (diluted 1:40 in PBS). Binding of cell marker was achieved within 30' at room temperature in a humid chamber. Coverslips were then washed three times by dipping in fresh PBS and drained. Afterwards coverslips were incubated with 40 ul fluorescene-conjugated rabbit anti-mouse immunoglobulin (Miles-Yeda), diluted 1:20 in PBS, for 30 minutes at room temperature. The coverslips were then washed in PBS and fixed in 5% acid/alcohol (5 ml of glacial acetic acid in 95 ml Ethanol) for 15 minutes at -20°C. They were then washed and drained as before.

Forty ul of rabbit anti-HSV (diluted 1:40 in PBS) was then added to each coverslip and incubated for 30' at room temperature, followed by another washing step. Coverslips were then incubated with 40 ul of goat anti-rabbit IgG conjugated to rhodamine, diluted 1:20 in PBS for 30 minutes.

Later, coverslips were washed 3 times in PBS then mounted in glycerol on glass slides, sealed with nail varnish and examined under a fluorescence microscope (Leitz Ortholux) and viewed with phase contrast optics or for immunofluorescence.

Temperature Sensitivity Assay of Recovered Viruses

Recovered viruses from latently infected mice were

assayed to determine if they retained their ts phenotype by titrating at both permissive and nonpermissive temperatures (31°C and 38.5°C) in the presence of EHu 5%.

Thermostability of HSV-2 ts Mutants

The ability of the wt HSV-2 and ts mutants to survive under explanted ganglia conditions was studied as follows.

Virus suspensions were prepared ($5x10^6$ - $5x10^7$ pfu/ml) in EFC50 medium. Virus suspensions were then aliquoted in one ml volumes in screwcapped vials. Vials were then incubated at 31°C and the titres determined at intervals of 0, 2, 4, 8, 12 hours; and 1, 2, 3, 4, 5, 6 & 7 days after incubation by plating 0.1 ml of each virus dilution onto two semiconfluent petri dishes of BHK21/C_{13} cells. After one hour of absorption at 38°C, two millilitres of EHu 5% were added to each petri dish. The cultures were then incubated at 31°C for 3 days before fixing and staining. The plaques were counted and the mean value of two different readings was recorded.

Homogenisation of Explanted Tissue:

Tissues from acutely infected mice

In the first group, the footpads were inoculated with a known concentration of HSV-2 (wt) before explantation. Then they were immediately explanted, homogenised and titrated (six footpads were used in this group).

In the second group, the footpads were explanted, then the virus was added before homogenisation and titration.

Tissues from Latently Infected Mice

Footpad tissues and dorsal root ganglia were explanted from latently infected female BALB/c mice that were inoculated with 10^6 pfu of HSV-2 wt virus/mouse.

Explanted tissues from individual footpads were

divided into two halves. The first half was homogenised in 0.15 ml of PBS + gentamicin + 10% CS (using glass homgeniser, Duall 20). The homogenate volume was increased to 0.5 ml by adding another 0.35 ml of PBS + gentamicin. The homogenate suspension was then titrated in duplicate on BHK cells. After one hour of absorption at 38.5°C, plates were overlaid with EHu 5% and incubated at 38.5°C for three days before they were fixed, stained and counted.

In each case, the other half of the footpad tissue, was cultured as before and the supernatant screened daily for released virus.

Explanted DRG (from the same mice) were also cultured and the supernatants screened as before except that the screening was performed 5-6 times a week instead of just twice a week.

Replication of HSV-2 wt and ts Mutants at Different Temperatures of Incubation

A rapid plaque assay was used to determine the ability of wt and ts mutants to grow at different temperatures of incubation. The selected temperatures were 31°C, 34.5°C, 36.5°C and 38.5°C.

Serial ten-fold dilutions of each virus stock were prepared in PBSCS. 0.1 ml of each dilution was allowed to absorb to BHK 21/C13 monolayers in 40 mm plates at 38.5°C for one hour. After absorption, 2 ml of EHu 5% were added to each plate. Plates were incubated for 3 days at the selected temperatures. Plates were then fixed, stained and the plaques were counted. The virus titre at each temperature was examined.

Histology

Dorsal root ganglia from healthy non-infected mice

were explanted and cultured with EFC50 for varying times. Cultured tissues were formalin fixed (for 24 hours). then embedded in paraffin, sectioned and hematoxyline/eosin stained. Sectioning and staining procedures were carried out in the Department of Pathology. Sections were studied by light microscopy.

Virus Particle Count - "Particle/pfu ratio"

This was done in the E.M. Unit in the Institute of Virology. The method used was that described by Watson (1962).

A sample of stock virus was diluted in PBSC to give approximately 10^8 pfu/ml. This was mixed with an equal volume of phosphotungstic acid (as negative stain) and the same volume of a suspension of latex beads at a known concentration. The latex beads were sonicated for 4-5 minutes before use. A sample of the mixture (3 ul) was spread onto a carbon grid, allowed to dry and viewed under the electron microscope (40.000X) magnification, the concentration of virus particles was calculated by comparing the number of virus particles present with the number of latex beads in a given field of the grid.

The number of particlesin the following virus stocks were counted: wt (HG52), ts 3, ts 5, ts 9 and ts 10.

Restriction Endonuclease Digestion of HSV DNA

Preparation of virus DNA

The method used was that described by Lonsdale (1979) with slight modifications.

BHK cells were seeded on Linbro wells at $5x10^5$ cells/well in 1 ml of GMEM - PIC medium. Linbro wells were then incubated at 37°C for 24 hours until a confluent monolayer formed.

After the medium was removed, each well was infected with 0.1 ml of virus suspension (m.o.i. 0.1 - 0.5 pfu/cell) and allowed to absorb for one hour at 38.5°C.

The cells were washed once (to remove unabsorbed viruses) with 700 ul of G.M.E.M. - P.I.C. after which 450 ul of fresh G.M.E.M. - P.I.C. was added to each well. Linbro wells were incubated at 32°C for 2 hours, before 50 uCi of ^{32}P-orthophosphate (Amersham, carrier-free) in 50 ul volume was added to each well and incubation continued at 32°C until an even cpe was evident (36-48 hours).

To harvest the infected cells, 500 ul of 5% (w/v) SDS was added directly to each well and mixed by swirling. The mixture in each well was transferred to a glass conical centrifuge tube and incubated at 37°C in a shaking water bath for 10 minutes. While standing in an ice bath, the transferred mixture was further mixed by inversion with 1 ml of saturated phenol. The tubes were inverted 10 times initially, then twice during the following 5' minutes to ensure sufficient mixing with the phenol.

The tubes were left to stand in the ice bath for 10 minutes before the phases were separated by centrifugation for 10 minutes at 2000 rpm (MSE cold spin) at +4°C. The aqueous phase (which contains the nucleic acid) was carefully removed to a glass centrifuge tube and the nucleic acid precipitated by the addition of 2 volumes (2 ml) ethanol (Analar), mixed by inversion 10 times and "pelleted" immediately by centrifugation at 2000 rpm (MSE cold spin) for 10 minutes at +4°C. The supernatant was discarded, the tubes were inverted to drain for 1-3 hours at room temperature.

The nucleic acids were resuspended in 0.2 ml of

distilled H_2O containing 20 ug of RNA-ase A, 50 units of RNA-ase T_1 and incubated in a shaking water bath at 37°C for 2 hours. Ten ul of the disolved nucleic acids were transferred to a 2.5 cm Whatman's no.1 filterpaper disc, and the remaining were transferred to Eppendorf tubes and stored at 4°C. The filter paper discs were air dried, then given three washes (5 minutes/each time) in 5% trichloroacetic acid, 2.5% sodium tetrapyrophosphate, then two washes in absolute ethanol and acetone. Finally, they were air dried before the labelled phosphate incorporated into DNA was counted in 5 ml of 5% toluene (P.P.O Koch-Light Laboratories).

Sample preparation

Immediately before using the samples for endonuclease digestion, the DNA samples were standardised by adding a suitable volume of water so as to have samples of equal RNA-ase resistance i.e. each sample containing approximately the same amount of radioactivity.

The endonuclease digestion was carried out in the following manner.

The required volumes of H_2O were first added to the wells of microtitre plates. This was followed by 6 ul of the appropriate reaction mixture (see Materials). The calculated volume of the prepared DNA was then added and this was followed by the endonuclease in a volume of 1 ul/sample.

Microtitre plates were sealed with sellotape and their contents mixed by swirling. Plates were then incubated at 38°C for 4-6 hours to allow the complete reaction to take place. The reactions were stopped by the addition of 5 ul/sample of R.E. Stop "Mixture".

Agarose Gel Electrophoresis

"Digests" were analysed by overnight or 24 hour agarose gel electrophoresis. Agarose at a particular strength was dissolved in 1xE buffer by boiling for 3 minutes (Microwave oven).

DNA samples were carefully loaded onto the gels and a constant voltage of 2 v/cm at room temperature was applied for approximately 12-24 hours. The agarose concentrations used were 0.6% for EcoRI, Hind III, Bgl II and Bam HI, while 0.8% agarose concentration was used for Kpn I.

Autoradiography

Gels were dried onto glass plates by standing overnight at 37°C (hot room) and then exposed to "Kodirex" film for 24-72 hours at -20°C. In some experiments, autoradiographic results were obtained by preflashing 'Kodak' XH1 film and exposing this in conjunction with an intensifier screen. Development of exposed films was performed in an X-Omat processor.

RESULTS

Mortality Following Peripheral Infection with Two Different Doses of wt Virus.

After footpad inoculation mice were scored daily (for the first 3 weeks) for signs of illness, paralysis of the hind legs, or death. Deaths within 24-48 hours or more than 4 weeks after inoculation were excluded.

The results of this experiment are shown in Table 6. No deaths or signs of illness were noticed in C57 BL/6-J, BALB/C or BIOZZI strain mice when they were inoculated with 10^5 pfu of HSV-2 (HG52 wt) virus. However 2 cases of paralysis followed by death were observed in strain "PIRBRIGHT" and 2 in strain "A", inoculated with the same dose. The paralysis was observed on the 18th day (PIRBRIGHT) and on the 11th day (A) post infection.

Apart from BIOZZI mice (where no paralysis or death took place at both doses), some paralysis and/or deaths were noticed in all other strains when the virus dose was increased to 10^6 pfu/mouse. In PIRBRIGHT mice, mortality increased from 8.3% (10^5 pfu) to 16.6% (10^6 pfu). Similarly, it increased from 0% to 27.7% in C57 BL/6-J and 0% to 10% in BALB/C. The highest mortality occurred with A/strain mice: 33.3% at 10^5 pfu and 58.3% at 10^6 pfu. No illness or death was observed before 8 days or after 21 days post infection. A shorter period of incubation was noticed in some mice inoculated with high doses.

Latency Establishment

Virus Reactivation From Latently Infected Mice

Virus released in the supernatant of footpad or dorsal root ganglia organ cultures was detected by screening the supernatant on semiconfluent BHK cells (grown in microtitre plates) at 31oC. The screening procedure, unless otherwise

TABLE 6

MICE MORTALITY FOLLOWING PERIPHERAL INFECTION WITH WT VIRUS

Mice Strains	10^5 pfu/ mouse No. of dead/ total	Mouse no.	Day of illness or death PI	%	10^6 pfu/ mouse No. of dead/ total	Mouse no.	Day of illness or death PI	%
Pirbright	2/24	1 2	18 18	8.3	3/18	1 2 3	9 18 13	16.6
C57BL/6-J	0/24	-	-	0	5/18	1 2 3 4 5	19 19 10 10 14	27.7
Balb/c	0/3	-	-	0	1/10	1	12	10
Biozzi	0/15	-	-	0	0/13	-	-	0
A	2/6	1 2	11 11	33.3	7/12	1 2 3 4 5 6 7	12 19 19 10 8 8 20	58.3

Virus was inoculated at 10^5 and 10^6 pfu/mouse into the rear footpad in 0.025 ml suspension.

PI Post infection

indicated, was carried out daily for the explanted footpad cultures and twice weekly for the DRG where the whole supernatant was removed and plated on BHK cells. Fresh medium was added to the cultures and incubation then continued (see Materials and Methods).

Any mouse where the footpad and/or one or more ganglia released virus (on one or more occasions) was scored as positive. The first screening always took place within 24-48 hours after explantation.

I. Reactivation of WT (HG52)

(Comparison of two different doses)

HSV-2 (wt) was inoculated at two doses of 10^5 pfu and 10^6 pfu/mouse to investigate the dose effect on latency.

A. Reactivation from Dorsal Root Ganglia (DRG).

Table 7 presents the recovery of wt virus from the DRG of latently infected mice. Using 10^5 pfu/mouse, the wt virus was successfully recovered from A, BALB/C and BIOZZI mice with a percentage of recovery of 100%, 66.6% and 84.6% respectively. From the PIRBRIGHT and C57 BL/6-J strains, virus could only be recovered from 60% and 40% of the mice inoculated. However, after initial inoculation with the higher dose of 10^6 pfu/mouse latency was established and the virus could be recovered from 100% of mice in all strains except C57 BL/6-J, where recovery was from 61.5%. The average time for virus reactivation (detection) was 15.5 -21.1 days after initial inoculation with 10^5 pfu/mouse, but this was decreased to 7.6 - 12.7 for the 10^6 pfu/mouse dose. The observed limits of variation in time of reactivation for each mouse strain is also given in Table 9.

B. Reactivation from the Footpad Tissues.

Attempts were made to reactivate the virus from

TABLE 7

RECOVERY OF WT VIRUS FROM THE DRG

Mice strains	10^5 pfu/mouse			10^6 pfu/mouse		
	Number of +ve/total	%	Reactivation time* (day)	Number of +ve/total	%	Reactivation time* (day)
Pirbright	9/15	60	15.8	6/6	100	11.5
C57 BL/6-J	4/10	40	15.5	8/13	61.5	12.7
Biozzi	11/13	84.6	21.1	6/6	100	11.3
Balb/c	2/3	66.6	18	6/6	100	7.6**
A	4/4	100	16.7	5/5	100	11

* Figures indicate the average time in days when the virus was recovered.
** The cultures were incubated and screened at 38°C. All others at 31°C.

extraneural tissue by explanting footpad tissues immediately after mouse death and supernatants were screened daily for released virus. Table 8 presents the wt virus recovery data from the footpad and also the average time when reactivation was first observed. With the exception of PIRBRIGHT and A/mice, the percentage of virus recovery in the three other strains was found to increase with increased input dose of virus. An increase in the initial dose of infection was correlated with subsequent reduction in the average time required for virus reactivation. This decreased from 23 days to 15.7 for C57 BL/6-J; from 43 days to 15 days for BIOZZI, and from 17 days to 9.7 days for BALB/C. Although the wt virus was recovered from the PIRBRIGHT mice when inoculated with the low dose of virus (3/22), yet, unexpectedly no virus was recovered after inoculation with the high dose (0/6). This result could be due to the small number of mice inoculated with the high dose of input virus. With the A/mice the footpad cultures of mice inoculated with $1x10^5$ pfu of virus were unfortunately lost through contamination.

Comparing the speed of recovery of wt virus following inoculation with 10^6 pfu/mouse, one finds recovery from the DRG as early as 7 days and as late as 16 days post explantation, while from the footpad it was first found at 7 days and latest at 27 days post explantation. Using 10^5 pfu of input virus, the FP tissues were first scored positive as early as 7 days and as late as 43 days post explantation while no significant changes were observed with the ganglia (Table 9).

C. Distribution of Reactivable wt Virus in DRG.

The distribution of wt virus recovery from different

TABLE 8

RECOVERY OF WT FROM THE FOOTPAD

Mice strain	10^5 pfu/mouse			10^6 pfu/mouse		
	Number of +ve/total	%	Reactivation time (day)*	Number of +ve/total	%	Reactivation time (day)*
Pirbright	3/22	13.6	22	0/6	0	(no isolate)
C57 BL/6-J	1/4	2.5	23	4/13	30.7	15.7
Biozzi	1/14	7.1	43	2/6	33.3	15
Balb/c	1/5	20	17	4/6	66.6	9.7**
A	L			3/5	60	11

* The average time (in days) on which the virus was recovered.
** Cultures were incubated and screened at 38°C.
L, Lost due to contamination

TABLE 9

THE AVERAGE TIME AT WHICH THE WT VIRUS WAS FIRST RECOVERED FROM THE DORSAL ROOT GANGLIA (DRG) AND FOOTPAD (FP)

wt pfu/ mouse	Pirbright		C57 BL/6-J		Biozzi		Balb/c		A	
	DRG	FP	DRG	FP	DRG	FP	DRG	FP	DRG	FP
10^5	[8-26] (15.8)	[18-24] (22)	[10-19] (15.5)	23**	[10-26] (21.1)	43**	18**	17**	[7-28] (16.7)	-
10^6	[10-13] (11.5)	- -	[9-16] (12.7)	[13-19] (15.7)	[10-12] (11.3)	[13-17] (15)	[7-8]* (7.6)	[8-12]* (9.7)	[10-15] (11)	[10-27] (15.6)

* Organ cultures were screened at 38.5°C.
** Virus recovered only on one occasion.
( ) Average time (mean time) in days after explantation.
[ ] The virus recovered as early as - and as late as - days after explantation.
\- No virus was recovered.

DRG of latently infected mice was investigated. Results are shown in Table 10, for virus used at 10^5 and 10^6 pfu doses.

Wt virus has never been recovered from ganglia explanted from the left side (contralateral to the site of inoculation). Virus was predominantly recovered from the lumbar ganglia particularly from 6th and 5th, but only rarely from the right side ganglia at the thoracic or sacral lumbar level.

At 10^6 pfu/mouse, the virus was very often recovered from lumbar, the 6th, 5th and 4th (22/31, 28/31, 17/31 respectively) while at 10^5 pfu/mouse the virus was predominantly recovered from these ganglia but to a lesser extent (15/30, 16/30, 16/30 respectively).

The pattern of spreading of wt virus to different ganglia differed between mouse strains. The virus was widely spreading in PIRBRIGHT and "A" strain mice where it reached the level of thoracic ganglia, while in the other three strains the virus was restricted to the lumbar region.

2. Reactivation of ts Mutant Viruses from Different Mouse Strains

Temperature-sensitive mutants were inoculated into the footpad of each of the five strains of mice. The capacity of these mutants to establish latent infection and to be recovered from the DRG or the FP were then checked. Tables 11, 13, 14, 16, 17, 19, 20, 22, 23, 25 show the data of ts mutant experiments.

PIRBRIGHT

Ts mutants were not efficiently recovered from the DRG of PIRBRIGHT strain mice (Table 11). No virus reactivated from the DRGs of mice inoculated with ts 2, 3, 5, 9, 10 and 11; with the exception of ts 7, 12 and 13 which were

TABLE 10

DISTRIBUTION OF REACTIVABLE WT VIRUS IN DRG OF LATENTLY INFECTED MICE (COMPARSION OF TWO DOSES)

Mice Strains	Dose pfu/ mouse	No. of positive mice/ total	DRG*										
			Sacral				Lumbar			Thoracic		Left Side	
			1st	6th	5th	4th	3rd	2nd	1st	13th	12th	1	2
Pirbright	10^5	9/15	4	7	8	5	5	2	2	3	1	0	0
	10^6	6/6	1	5	6	3	2	2	2	0	0	0	0
C57BL/6-J	10^5	4/10	2	2	0	1	0	0	0	0	0	0	0
	10^6	8/13	1	5	6	4	0	0	0	0	0	0	0
Biozzi	10^5	11/13	1	1	5	6	2	1	0	0	0	0	0
	10^6	6/6	0	3	5	3	0	0	0	0	0	0	0
Balb/c	10^5	2/3	0	2	1	1	0	0	0	0	0	0	0
	10^6	6/6	1	4	6	4	3	0	1	0	0	0	0
A	10^5	4/4	0	3	2	3	2	2	2	2	0	0	0
	10^6	5/5	1	5	5	3	1	3	0	3	3	0	0
TOTAL	10^5	30/45	7	15	16	16	9	5	4	5	1		
	10^6	31/35	4	22	28	17	6	5	3	3	3		

* Figures indicate the number of times at which particular ganglia released virus. A single mouse may have one or more ganglia releasing virus.

recovered from a signficant number of mice (40%, 21.4% and 18.1% respectively), the remaining mutants were rarely recoverable.

No signficant sex related difference was detected with regard to reactivation from latency. The distribution of the ts mutants within the DRG of the PIRBRIGHT mice was restricted to the 6th and 5th lumbar ganglia (Table 12).

Recovery of the ts mutants from the footpad tissues of PIRBRIGHT mice was even lower (Table 13). Only 4 ts mutants (ts 2, 3, 12 and 13), were ever recovered from cultured footpads, and these only on one occasion each.

On average ts mutants established latency in PIRBRIGHT mice, in the DRG, in 8.5% of individuals (Table 11) and in the footpad in 2.5% of individuals (Table 13).

C57 BL/6-J

The reactivation of the thirteen ts mutants from the DRG of C57 BL/6-J mice is presented in Table 14. Five of the mutants were not recovered (ts 2, 7, 9, 10 and 13). Ts 1 and ts 12 were recovered from 41.6% and 45.4% of mice with mutants ts 3, 4, 5, 6, 8 and 11 less often successful (8.3%, 30.7%, 8.3%, 16.6%, 30.7% and 7.6% respectively). It seems that C57BL/6J mice are more susceptible (14.7%) than PIRBRIGHT mice to ts mutant induced latent infection in DRG. The mutants ts 2, 3, 4, 6, 7, 8, 9 and 10 were also recovered from the footpad tissue of C57BL/6J mice (Table 15). Ts 2, 9 and 12 reasonably frequently (25%, 23% and 30% respectively). The average ts mutant recovery from the footpad of this mouse strain was 9.6%.

The spread of the ts mutants in the DRG of the C57 BL/6-J mice was also restricted. Although some mutants were recovered occasionally from the first sacral ganglion,

TABLE 11

RECOVERY OF TS MUTANTS FROM THE DRG OF PIRBRIGHT MICE

Virus	Dose pfu/ mouse	No. of +ve/total Female	Male	Cumulative data	%
ts1	$1x10^5$	0/9	1/8	1/17	5.8
ts2	$1.7x10^6$	0/7	0/7	0/14	0
ts3	$1.2x10^5$	0/5	0/9	0/14	0
ts4	$5x10^6$	1/4	1/11	2/15	13.3
ts5	$1.7x10^6$	0/7	0/6	0/13	0
ts6	$1x10^6$	0/11	1/4	1/15	6.6
ts7	$8x10^5$	3/5	3/10	6/15	40
ts8	$2.5x10^5$	1/8	0/6	1/14	7.1
ts9	$7.5x10^5$	0/9	0/10	0/19	0
ts10	$2.5x10^5$	0/6	0/9	0/15	0
ts11	$4.2x10^5$	0/1	0/11	0/12	0
ts12	$5x10^5$	2/6	1/8	3/14	21.4
ts13	$6x10^5$	1/5	1/6	2/11	18.1
TOTAL		8/83 (9.6%)	8/105 (7.6%)	16/188 (8.5%)	

TABLE 12

DISTRIBUTION OF REACTIVABLE TS MUTANTS IN DRG OF PIRBRIGHT MICE*

Virus	Dose/pfu mouse	No. of +ve mice	Lumbar 6	5	4	3	2	1	Thoracic 13,	12	left side 1,	2	1st sacral
ts1	$1x10^5$	1	1	0	0	0	0	0	0	0	0	0	0
ts4	$5x10^6$	2	1	1	0	0	0	0	0	0	0	0	0
ts6	$1x10^6$	1	0	1	0	0	0	0	0	0	0	0	0
ts7	$8x10^5$	6	4	2	0	0	0	0	0	0	0	0	0
ts8	$2.5x10^5$	1	0	1	0	0	0	0	0	0	0	0	0
ts12	$5x10^5$	3	1	2	0	0	0	0	0	0	0	0	0
ts13	$6x10^5$	2	1	1	0	0	0	0	0	0	0	0	0
TOTAL		16	8	8									

* Only those mutants recoverable from the DRG are listed in this Table.

TABLE 13

RECOVERY OF TS MUTANTS FROM THE FOOTPAD OF PIRBRIGHT MICE

Virus	Dose pfu/mouse	No. of +ve/total Female	Male	Cumulative data	%
ts1	$1x10^5$	0/6	0/5	0/11	0
ts2	$1.7x10^6$	0/6	1/5	1/11	9
ts3	$1.2x10^5$	0/3	1/3	1/6	16.6
ts4	$5x10^6$	0/4	0/4	0/15	0
ts5	$1.7x10^6$	0/7	0/6	0/13	0
ts6	$1x10^6$	0/10	0/4	0/14	0
ts7	$8x10^5$	0/6	0/11	0/17	0
ts8	$2.5x10^5$	0/7	0/5	0/12	0
ts9	$7.5x10^5$	0/6	0/6	0/12	0
ts10	$2.5x10^5$	0/5	0/6	0/11	0
ts11	$4.2x10^5$	0/1	0/11	0/12	0
ts12	$5x10^5$	1/5	0/7	1/12	8.3
ts13	$6x10^5$	0/4	1/5	1/9	11.1
TOTAL		1/70 (1.4%)	3/85 (3.5%)	4/155 (2.5%)	

TABLE 14

RECOVERY OF TS MUTANTS FROM THE DRG OF C57BL/6-J MICE

Virus	Dose pfu/mouse	No. of +ve/total Female	Male	Cumulative data	%
ts1	$1x10^5$	2/3	3/9	5/12	41.6
ts2	$1.7x10^6$	0/8	0/6	0/14	0
ts3	$1.2x10^5$	0/5	1/7	1/12	8.3
ts4	$5x10^6$	3/8	1/5	4/13	30.7
ts5	$1.7x10^6$	0/6	1/6	1/12	8.3
ts6	$1x10^6$	1/3	1/9	2/12	16.6
ts7	$8x10^5$	0/2	0/10	0/12	0
ts8	$2.5x10^5$	3/6	1/7	4/13	30.7
ts9	$7.5x10^5$	0/7	0/6	0/13	0
ts10	$2.5x10^5$	0/6	0/6	0/12	0
ts11	$4.2x10^5$	1/9	0/4	1/13	7.6
ts12	$5x10^5$	3/5	2/6	5/11	45.4
ts13	$6x10^5$	0/5	0/2	0/7	0
TOTAL		13/73 (17.8%)	10/83 (12%)	23/156 (14.7%)	

TABLE 15

RECOVERY OF TS MUTANTS FROM THE FOOTPAD TISSUE OF C57BL/6-J MICE

Virus	Dose pfu/mouse	No. of +ve/total Female	Male	Cumulative data	%
ts1	$1x10^5$	0/3	0/8	0/11	0
ts2	$1.7x10^6$	1/6	1/2	2/8	25
ts3	$1.2x10^5$	1/4	0/5	1/9	11.1
ts4	$5x10^6$	1/8	0/5	1/13	7.6
ts5	$1.7x10^6$	0/6	0/6	0/12	0
ts6	$1x10^6$	0/3	1/9	1/12	8.3
ts7	$8x10^5$	0/4	1/6	1/10	10
ts8	$2.5x10^5$	0/1	1/5	1/6	16.6
ts9	$7.5x10^5$	1/7	2/6	3/13	23
ts10	$2.5x10^5$	0/6	0/6	0/12	0
ts11	$4.2x10^5$	0/8	0/4	0/12	0
ts12	$5x10^5$	2/5	1/5	3/10	30
ts13	$6x10^5$	0/5	0/2	0/7	0
TOTAL		6/66 (9%)	7/69 (10.1%)	13/135 (9.6%)	

none of the mutants were recovered from a ganglion beyond the 4th lumbar. The ganglia most frequently releasing virus were again the 5th and 6th lumbar (Table 16). There was no sex dependent difference in reactivation from latency.

BIOZZI

In this strain four mutants (ts 1, 2, 4 and 8) reactivated with >33% efficiency (36.3%, 47%, 58.7% and 35%) following the establishment of latent infection in their DRG. Four other mutants (ts 6, 7, 11 and 13) were less frequently reactivated and recovered, but mutants ts 3, 5, 9, 10 and 12 were never recovered from the DRG of this strain of mice. The average recovery of mutants was 19.6% (Table 17). No significant difference in reactivation behaviour was found between males and females. The mutants behaved differently as far as their rate of recovery from the footpad was concerned (Table 18). Most frequently recovered was ts 3 where 5/15 of the cultured footpad tissues released virus while all ts 3 infected DRG explants remained negative. Mutants ts 1, 2, 4, 6, 7, 8, 9 and 12 were also recovered from the footpad but only on one or two occasions in each case. On average, ts mutant recovery from BIOZZI footpad tissue was 9.2%.

No virus was recovered from ganglia beyond the 4th lumbar, and the pattern of ts mutants spread through the (BIOZZI) ganglia was similar to that observed in PIRBRIGHT and C57BL/6J mice (Table 19).

BALB/C

Seven of the temperature sensitive mutants (ts 1, 4, 7, 8, 10, 11 and 12) were more than 33% efficient in their reactivation from the latent infection in the DRG of BALB/C mice and only 4 mutants (ts 3, 5, 9 and 13) were never

TABLE 16

DISTRIBUTION OF REACTIVABLE TS MUTANTS IN DRG OF C57BL/6-J MICE*

Virus	pfu/mouse	No. of +ve mice	1st sacral	Lumbar						Thoracic left side			
				6	5	4	3	2	1	13,	12	1,	2
ts1	$1x10^5$	5	1	1	3	2	0	0	0	0	0	0	0
ts3	$1.2x10^5$	1	0	1	1	0	0	0	0	0	0	0	0
ts4	$5x10^6$	4	1	1	2	3	0	0	0	0	0	0	0
ts5	$1.7x10^6$	1	0	0	1	0	0	0	0	0	0	0	0
ts6	$1x10^6$	2	0	1	1	0	0	0	0	0	0	0	0
ts8	$2.5x10^5$	4	0	1	2	1	0	0	0	0	0	0	0
ts11	$4.2x10^5$	1	0	0	1	0	0	0	0	0	0	0	0
ts12	$5x10^5$	5	2	2	3	0	0	0	0	0	0	0	0
TOTAL		23	4	7	14	6							

* Only those mutants recoverable from the DRG are listed in this Table.

TABLE 17

RECOVERY OF TS MUTANTS FROM THE DRG OF BIOZZI STRAIN OF MICE

Virus	Dose pfu/mouse	No. of +ve/total Female	Male	Cumulative data	%
ts1	$1x10^5$	1/2	3/9	4/11	36.3
ts2	$1.7x10^6$	4/6	4/11	8/17	47
ts3	$1.2x10^5$	0/4	0/12	0/16	0
ts4	$5x10^6$	5/6	7/8	12/14	85.7
ts5	$1.7x10^6$	0/5	0/7	0/12	0
ts6	$1x10^6$	1/7	0/9	1/16	6.2
ts7	$8x10^5$	1/4	1/10	2/14	14.2
ts8	$2.5x10^5$	2/6	3/8	5/14	35.7
ts9	$7.5x10^5$	0/5	0/6	0/11	0
ts10	$2.5x10^5$	0/4	0/9	0/13	0
ts11	$4.2x10^5$	0/4	1/8	1/12	8.3
ts12	$5x10^5$	0/6	0/8	0/14	0
ts13	$6x10^5$	1/5	1/9	2/14	14.2
TOTAL		15/64 (23.4%)	20/114 (17.5%)	35/178 (19.6%)	

TABLE 18

RECOVERY OF TS MUTANTS FROM THE FOOTPAD OF BIOZZI MICE

Virus	Dose pfu/mouse	No. of +ve/total Female	Male	Cumulative data	%
ts1	$1x10^5$	0/2	2/8	2/10	20
ts2	$1.7x10^6$	0/6	1/10	1/16	6.2
ts3	$1.2x10^5$	0/2	5/13	5/15	33.3
ts4	$5x10^6$	0/7	1/7	1/14	7.1
ts5	$1.7x10^6$	0/5	0/7	0/12	0
ts6	$1x10^6$	1/7	0/9	1/16	6.2
ts7	$8x10^5$	1/5	0/6	1/11	9
ts8	$2.5x10^5$	0/5	1/4	1/9	11.1
ts9	$7.5x10^5$	0/4	2/7	2/11	18.1
ts10	$2.5x10^5$	0/4	0/5	0/9	0
ts11	$4.2x10^5$	0/4	0/8	0/12	0
ts12	$5x10^5$	1/6	0/8	1/14	7.1
ts13	$6x10^5$	0/5	0/9	0/14	0
TOTAL		3/62 (4.8%)	12/101 (11.8%)	15/163 (9.2%)	

TABLE 19

DISTRIBUTION OF REACTIVABLE TS MUTANTS IN DRG OF BIOZZI MICE*

Virus	pfu/mouse	No. of +ve mice	1st sacral	Lumbar						Thoracic		left side	
				6	5	4	3	2	1	13,	12	1,	2
ts1	$1x10^5$	4	0	1	2	1	0	0	0	0	0	0	0
ts2	$1.7x10^6$	8	2	4	3	2	0	0	0	0	0	0	0
ts4	$5x10^6$	12	2	5	6	4	0	0	0	0	0	0	0
ts6	$1x10^6$	1	0	0	0	1	0	0	0	0	0	0	0
ts7	$8x10^5$	2	0	0	2	2	0	0	0	0	0	0	0
ts8	$2.5x10^5$	5	0	3	2	0	0	0	0	0	0	0	0
ts11	$4.2x10^5$	1	0	1	0	0	0	0	0	0	0	0	0
ts13	$6x10^5$	2	0	2	1	0	0	0	0	0	0	0	0
TOTAL		35	4	16	16	10							

* Only these mutants recoverable from the DRG are listed in this Table.

recovered. The average ts mutant recovery was 27.4% (Table 20). Among all of the ts mutants used ts 8 was recovered from 8/9 mice. Recovery from the footpad was also good with five ts mutants (ts 1, 2, 4, 8 and 10) being successfully isolated from a third or more of the inoculated mice. Only three mutants ts 5, ts 11 and ts 12 were not recovered from the footpad of BALB/C mice (Table 21). Average ts mutant reisolation from footpad tissues was 22.6%.

Although the ts mutants were restricted to the lumbar ganglia in their distribution, both ts 1 and ts 4 were reactivated and recovered from the 3rd lumbar ganglion and ts 1, 4, 11 and 12 were also recovered from the 1st sacral ganglion (Table 22).

Strain "A"

The DRG latency negative group of ts mutants in "A" strain mice consist of ts 3, 5 and 7 (Table 23). All other mutants were demonstrated to establish latent infection in the DRG of this strain of mice bringing the average efficiency to 25%. According to their efficiencies, the mutants can be listed in a decreasing order as ts 2, 8, 1, 4, 12, 6, 10, 11, 13 and 9. The differences between males and females observed in this strain remain to be confirmed. The results of ts mutant recovery from the footpad are shown in Table 24. Eight mutants were reisolated from footpad explants. Three mutants were recovered from a third or more of the footpads, namely ts 4, ts 10 and ts 3. The mutants that could not be reisolated were ts 1, 5, 7, 9 and 11. The average percentage of recovery of ts mutants reached 18.7% in this particular strain of mice.

No virus has been isolated from the explanted 1st

TABLE 20

RECOVERY OF TS MUTANTS FROM THE DRG OF BALB/C MICE

Virus	Dose pfu/mouse	No. of +ve/total Female	Male	Cumulative data	%
ts1	$1x10^5$	2/5	6/8	8/13	61.5
ts2	$1.7x10^6$	1/8	0/5	1/13	7.6
ts3	$1.2x10^5$	0/6	0/8	0/14	0
ts4	$5x10^6$	6/14	5/13	11/27	40.7
ts5	$1.7x10^6$	0/7	0/4	0/11	0
ts6	$1x10^6$	1/8	0/7	1/15	6.6
ts7	$8x10^5$	2/6	3/9	5/15	33.3
ts8	$2.5x10^5$	8/9	-	8/9	88.8
ts9	$7.5x10^5$	0/4	0/7	0/11	0
ts10	$2.5x10^5$	2/6	2/6	4/12	33.3
ts11	$4.2x10^5$	1/5	3/6	4/11	36.3
ts12	$5x10^5$	4/7	1/3	5/10	50
ts13	$6x10^5$	0/8	0/2	0/10	0
TOTAL		27/93 (29%)	20/78 (25.6%)	47/71 (27.4%)	

TABLE 21

RECOVERY OF TS MUTANTS FROM THE FOOTPAD OF BALB/C MICE

Virus	Dose pfu/mouse	No. of +ve/total Female	Male	Cumulative data	%
ts1	$1x10^5$	3/5	2/7	5/12	41.6
ts2	$1.7x10^6$	2/6	-	2/6	33.3
ts3	$1.2x10^5$	3/5	0/6	3/11	27.2
ts4	$5x10^6$	2/6	1/3	3/9	33.3
ts5	$1.7x10^6$	0/7	0/4	0/11	0
ts6	$1x10^6$	1/9	0/6	1/15	6.6
ts7	$8x10^5$	1/5	1/5	2/10	20
ts8	$2.5x10^5$	4/9	-	4/9	44.4
ts9	$7.5x10^5$	1/3	1/6	2/9	22.2
ts10	$2.5x10^5$	2/6	4/6	6/12	50
ts11	$4.2x10^5$	0/3	0/7	0/10	0
ts12	$5x10^5$	0/1	0/3	0/4	0
ts13	$6x10^5$	1/8	0/2	1/10	10
TOTAL		20/73 (27.3%)	9/55 (16.3%)	29/128 (22.6%)	

TABLE 22

DISTRIBUTION OF REACTIVABLE TS MUTANTS IN DRG OF BALB/C MICE*

Virus	pfu/mouse	No. of +ve mice	1st sacral	Lumbar						Thoracic		left side	
				6	5	4	3	2	1	13,	12	1,	2
ts1	$1x10^5$	8	1	2	6	4	2	0	0	0	0	0	0
ts2	$1.7x10^6$	1	0	1	0	0	0	0	0	0	0	0	0
ts4	$5x10^6$	11	1	3	7	3	1	0	0	0	0	0	0
ts6	$1x10^6$	1	0	0	1	0	0	0	0	0	0	0	0
ts7	$8x10^5$	5	0	3	5	1	0	0	0	0	0	0	0
ts8	$2.5x10^5$	8	0	4	7	1	0	0	0	0	0	0	0
ts10	$2.5x10^5$	4	0	3	1	2	0	0	0	0	0	0	0
ts11	$4.2x10^5$	4	1	3	1	1	0	0	0	0	0	0	0
ts12	$5x10^5$	5	1	2	3	0	0	0	0	0	0	0	0
TOTAL		47	4	21	31	12	3						

* Only those mutants recoverable from the DRG are listed in this Table.

TABLE 23

RECOVERY OF TS MUTANTS FROM THE DRG OF "A" STRAIN OF MICE

Virus	Dose pfu/mouse	No. of +ve/total Female	Male	Cumulative data	%
ts1	$1x10^5$	6/9	1/4	7/13	53.8
ts2	$1.7x10^6$	8/9	-	8/9	88.8
ts3	$1.2x10^5$	0/8	0/16	0/24	0
ts4	$5x10^6$	2/6	4/9	6/15	40
ts5	$1.7x10^6$	0/6	0/5	0/11	0
ts6	$1x10^6$	2/5	0/3	2/8	25
ts7	$8x10^5$	0/4	0/9	0/13	0
ts8	$2.5x10^5$	7/8	-	7/8	87.5
ts9	$7.5x10^5$	0/6	1/6	1/12	8.3
ts10	$2.5x10^5$	1/1	1/9	2/10	20
ts11	$4.2x10^5$	1/10	1/3	2/13	15.3
ts12	$5x10^5$	3/8	1/5	4/13	30.7
ts13	$6x10^5$	0/5	1/6	1/11	9
TOTAL		30/85 (35.2%)	10/75 (13.3%)	40/160 (25%)	

TABLE 24

RECOVERY OF TS MUTANTS FROM THE FOOTPAD OF "A" STRAIN OF MICE

Virus	Dose pfu/mouse	No. of +ve/total Female	Male	Cumulative data	%
ts1	$1x10^5$	0/8	0/3	0/11	0
ts2	$1.7x10^6$	2/9	-	2/9	22.2
ts3	$1.2x10^5$	2/5	3/10	5/15	33.3
ts4	$5x10^5$	2/5	6/9	8/14	57.1
ts5	$1.7x10^6$	0/6	0/5	0/11	0
ts6	$1x10^6$	2/7	1/6	3/13	23.0
ts7	$8x10^5$	0/3	0/8	0/11	0
ts8	$2.5x10^5$	1/8	-	1/8	12.5
ts9	$7.5x10^5$	0/4	0/4	0/8	0
ts10	$2.5x10^5$	0/1	5/9	5/10	50
ts11	$4.2x10^5$	0/8	0/4	0/12	0
ts12	$5x10^5$	1/6	0/5	1/11	9
ts13	$6x10^5$	2/5	0/6	2/11	18.1
TOTAL		12/75 (16%)	15/69 (21.7%)	27/144 (18.7%)	

sacral ganglion, but mutants ts 2 and ts 4 have been recovered from the 3rd lumbar ganglion. Reactivation was never observed from ganglia other than those of the lumbar region (Table 25).

In addition to the generally lower capacity to induce latency, the ts mutants were also found to need a longer time for reactivation compared to that of the wild type. The average time of reactivation of the mutants from either sites (DRG and FP) of different mouse strains is presented in Table 26. In general, ts mutant viruses are slower to reactivate from the footpad than from the ganglia.

3. Reactivation of wt virus from the Brain and Spinal Cord of Peripherally Inoculated Mice

Brain or spinal cord tissues from mice that had survived for three months after being infected with HSV-2 wt (10^5-10^6 pfu per mouse) were explanted and cocultivated with HeLu cells at 31°C. Slight cell toxicity was observed in some cultures when the spinal cord or brain pieces were cocultivated.

The results of the experiment show that the wt virus can be recovered from the spinal cord, but not from the brain, of "A" strain of mice. No virus was recovered either from the brain or from the spinal cord of PIRBRIGHT, C57BL/J, BIOZZI mice even though it was recovered from the DRG of all but one of the animals tested (Table 27).

One of the two mice that released virus from their spinal cord also shed virus from the footpad.

4. Behaviour of HSV-2 (wt) in a Paralysed and in a Clinically Normal Latently Infected Mouse

Attempts were made to reactivate HSV-2 (wt) from different sites of paralysed and non-paralysed mice.

TABLE 25

DISTRIBUTION OF REACTIVABLE TS MUTANTS IN DRG OF "A" STRAIN OF MICE*

Virus	pfu/mouse	No. of +ve mice	1st sacral	Lumbar						Thoracic		left side	
				6	5	4	3	2	1	13	12	1	2
ts1	$1x10^5$	7	0	4	5	1	0	0	0	0	0	0	0
ts2	$1.7x10^6$	8	0	1	7	5	1	0	0	0	0	0	0
ts4	$5x10^6$	6	0	3	4	2	2	0	0	0	0	0	0
ts6	$1x10^6$	2	0	1	1	0	0	0	0	0	0	0	0
ts8	$2.5x10^5$	7	0	4	7	2	0	0	0	0	0	0	0
ts9	$7.5x10^5$	1	0	0	1	0	0	0	0	0	0	0	0
ts10	$2.5x10^5$	2	0	1	0	1	0	0	0	0	0	0	0
ts11	$4.2x10^5$	2	0	0	1	1	0	0	0	0	0	0	0
ts12	$5x10^5$	4	0	1	3	0	0	0	0	0	0	0	0
ts13	$6x10^5$	1	0	1	1	0	0	0	0	0	0	0	0
TOTAL		40	0	16	30	12	3						

* Only DRG +ve viruses are listed in this Table.

TABLE 26

TIME OF REACTIVATION OF TS MUTANTS VIRUSES FROM FP AND DRG OF DIFFERENT STRAINS OF MICE

Virus	pfu/ mouse	Mouse Strains											
		PIRBRIGHT		C57BL/J		BIOZZI		BALB/c		A		Overall Extremes	
		DRG	FP	DRG	FP	DRG	FP	DRG	FP	DRG	FP	DRG	FP
ts1	$1x10^5$	16	-	11-21 (18.2)	-	14-17 (15.2)	14-21 (17.5)	15-23 (18.8)	17-32 (24.8)	11-20 (15.8)	-	11-23	14-32
ts2	$1.7x10^6$	-	44	-	28.5	17-23 (19.2)	23	23	24	14-16 (15.2)	17-21 (19)	14-23	17-44
ts3	$1.2x10^5$	-	34	28	36	-	10-25 (15.8)	-	11-20 (14)	-	14-16 (14.6)	28	10-36
ts4	$5x10^6$	20	-	10-20 (17)	14	10-20 (16.2)	37	14-18 (17)	18-27 (20.7)	11-16 (13.1)	12-19 (15.8)	10-20	12-37
ts5	$1.7x10^6$	-	-	23	-	-	-	-	-	-	-	23	-
ts6	$1x10^6$	17	-	19-23 (21)	16	19	30	13	19	13-28 (20.5)	20-24 (22.6)	13-28	16-30
ts7	$8x10^5$	16-22 (17)	-	-	23	16-22 (19)	37	-	14	-	-	16-22	14-37
ts8	$2.5x10^5$	21	-	8-15 (12.3)	15	11-19 (14.8)	14	11-14 (11.3)	12-22 (16)	14	18	8-21	12-22
ts9	$7.5x10^5$	-	-	-	19-25 (22.3)	-	12-16 (14)	-	23	25	-	25	12-25
ts10	$2.5x10^5$	-	-	-	-	-	-	12-19 (17.2)	15-26 (21.6)	19	13-45 (20.2)	12-19	13-45
ts11	$4.2x10^5$	-	-	14	-	14	-	17-25 (19.2)	-	14-25 (14.5)	-	14-25	-
ts12	$5x10^5$	17	8	17-18 (17.4)	8-21 (15.3)	-	23	14	-	10-16 (14)	15	10-18	8-23
ts13	$6x10^5$	14-18 (16)	20	-	-	11-14 (12.5)	-	-	18	18-24 (22)	13-18 (15.5)	8-24	13-20
Overall extremes (strains)		14-21	8-44	8-28	8-36	10-23	10-37	11-25	11-32	10-28	12-45		

- no virus recovered

x - y earliest and latest time of virus recovery; ( ) average time of reactivation

Single numbers indicate that virus was recovered in one occasion only.

TABLE 27

REACTIVATION OF HSV-2 (WT) FROM SPINAL CORD OF LATENTLY INFECTED MICE

Mice Strain	Dose pfu/mouse	No. of mice used	No. of positives			
			DRG	FP	SP	BR
Pirbright	10^5	2	2	0	0	0
C57BL/6-J	10^5	4	3	1	0	0
Biozzi	10^5	3	3	1	0	0
A	10^6	5	5	3	2	0

DRG dorsal root ganglia
FP footpad
SP spinal cord
BR brain

HSV-2 (wt) was inoculated into the footpad of male PIRBRIGHT mice at a dose of 10^6 pfu/mouse. Mice were left to survive the infection and recover. Many mice appeared well again and free of overt signs of infection. However, some mice (3/18) were showing persistent limb paralysis, one of the latter mice was left to survive for another 5 months after inoculation (Figure 18). Subsequently mice were killed with chloroform and their left and right footpads, sciatic nerves, dorsal root ganglia and the spinal cord and the brain were explanted, cultured and screened for reactivated virus.

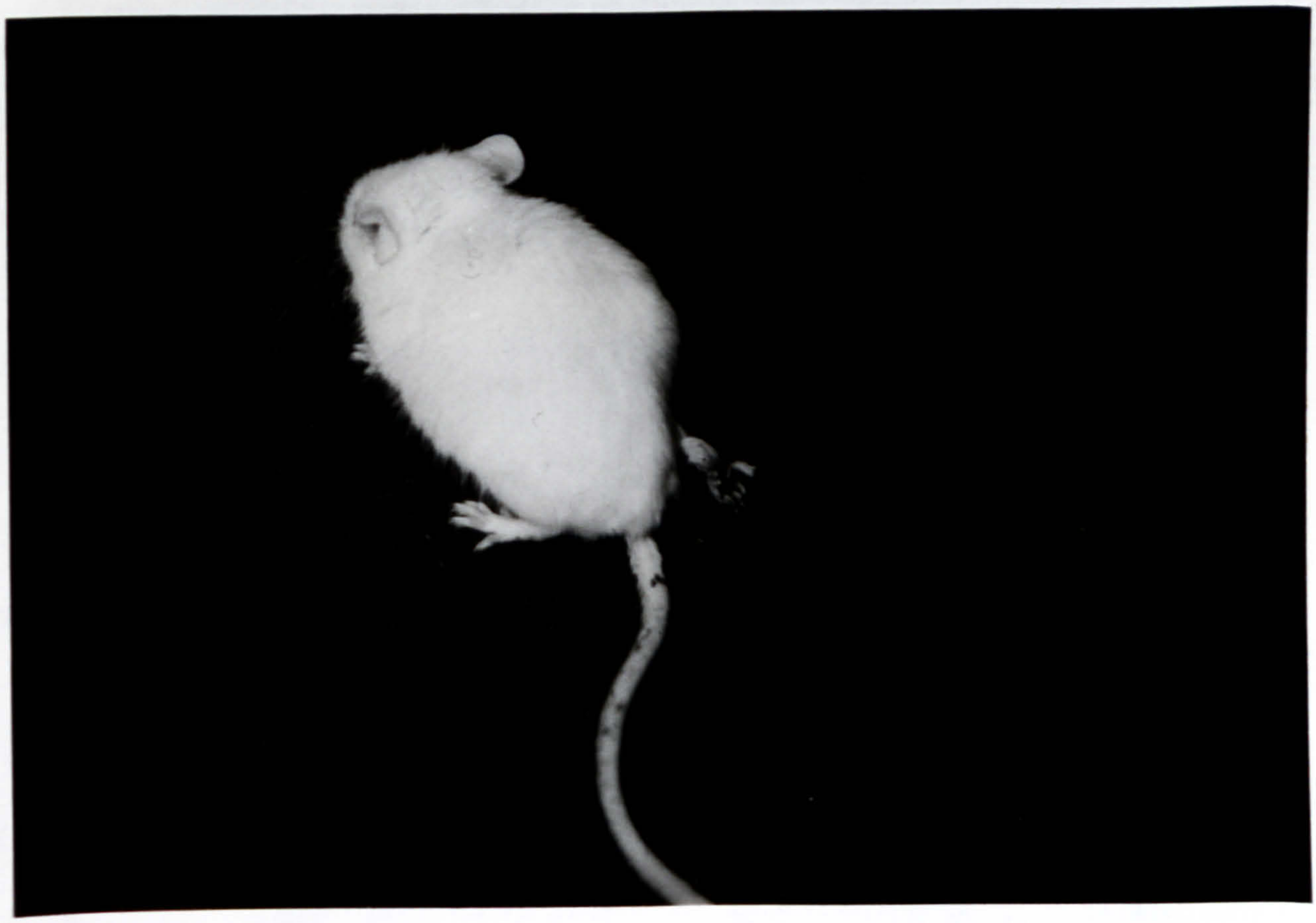
In the latently infected, non-paralysed mouse wt virus proved recoverable from right side sensory ganglia only and there only from the 5th and 6th lumbar (Table 28).

In the mouse with the paralysed right rear limb, the virus proved recoverable not only from six right side ganglia but also from the left side 6th and 5th lumbar ganglia and from the spinal cord. Virus reactivation was first observed from the right side DRG of the paralysed mouse ten days after explantation. Three days later the left side ganglia and the spinal cord released virus and at the same time virus was first recovered from the DRG of the non-paralysed mouse. Virus was not recovered from the footpads, sciatic nerves or brains of these two mice.

5. Latency Establishment and Reactivation of ts Mutants Viruses in the Mouse Brain

The ability of some of the HSV-2 ts mutants to establish latent infection and to be recovered from BALB/C mouse brains was studied. The mutants chosen had been found to be either non recoverable (ts 3, ts 5 and ts 9) or the least recoverable (ts 6) from the DRG of latently infected

FIGURE 18



Persistent hind limb (right) paralysis in PIRBRIGHT mouse 5 months after footpad inoculation with 10^6 pfu of HSV-2 strain HG52.

TABLE 28

RECOVERY OF HG52 (WT) VIRUS FROM PARALYSED AND FROM CLINICALLY NORMAL (LATENTLY INFECTED) MICE*

Mouse Condition	Footpad	Sciatic Nerve	DRG Sacral I	DRG Lumbar 6	DRG Lumbar 5	DRG Lumbar 4	DRG Lumbar 3	DRG Lumbar 2	DRG Lumbar 1	DRG Thoracic 13	DRG Thoracic 12	Spinal Cord	Brain**
Latently Infected, (Clinically Normal)	R	-	-	+	+	-	-	-	-	-	-	-	-
	L	-	-	-	-	-	-	-	-	-	-		
Latently Infected, (Paralysis)	R	-	-	+	+	+	+	+	+	-	-	+	-
	L	-	-	+	+	-	-	-	-	-	-		

* Strain Pirbright
** Brain cultures were discarded 15 days post incubation due to contamination
R Right side
L Left side

BALB/C mice. The mutants were inoculated at a dose of 10^5 and 10^6 pfu/mouse in 0.05 ml intracerebrally. Six weeks later brain tissues were explanted and cocultivated as small pieces with HeLu cells (one piece of tissue cultivated in each petri dish) at 31°C. Some of the brain pieces were cultured alone with EFC50 as an organ culture and the supernatants screened for released virus (see Materials and Methods). Cultures were screened for any cytopathic effect.

No virus was recovered from the mouse brains inoculated with ts 3, ts 5 or ts 9 at either dose even after 25 days of incubation, and even when the cultures (brain pieces and HeLu cells) were sonicated and replated on Cl3 cells. However, one cocultivated brain tissue from the ts 6 inoculated mouse released virus after 23 days of co-cultivation. Moreover, when tissues and cells from the ts 6 inoculated mice were harvested, sonicated and replated on Cl3 cells, another mouse brain tissue was found to have released virus (Table 29).

A normal, uninoculated, mouse brain and virus inoculated HeLu cell culture were used as controls respectively. The viruses isolated proved to be still temperature sensitive.

6. Immunofluorescence Studies and Virus Reactivation From Dissociated Ganglia in Culture

Left and right side dorsal root ganglia from mice that had survived for three months after being infected were explanted, dissociated and cultured at either 31°C or 37°C. Ganglia from 3-4 mice were pooled and dissociated in each experiment. The supernatants of the DRG cell cultures were sampled (100 ul) daily and plated on BHK21/Cl3 cell cultures. Virus reactivation was recognised by the

TABLE 29

LATENT INFECTION AND RECOVERY OF TS MUTANTS FROM MOUSE BRAIN*

Virus	Dose pfu/mouse	No. of +ve/total
ts 3	10^5	0/7
ts 5	10^5	0/7
ts 5	10^6	0/4
ts 6	10^5	2/13**
ts 9	10^5	0/10
ts 9	10^6	0/5

* 3 week old male and female BALB/c mice were used.

** One isolate was recovered 23 days after co-cultivation; the other isolate was recovered on the 32nd day and only after harvesting
and sonication of the cultured cells.

appearance of cpe either in BHK21/C13 cells or in the dissociated DRG cultures.

The wt virus was successfully reactivated from dissociated right side DRG of PIRBRIGHT mice (Table 30), though reactivation of the virus from two other strains (BALB/C and BIOZZI) were not achieved. The likely explanation for this observation is that mice of the latter two strains have been used only in one experiment each, while four dissociation experiments were carried out using PIRBRIGHT mice.

In addition, attempts to reactivate ts 3 from dissociated DRG cultures of "A" strain mice at 31°C were also carried out. No virus was recovered and no positive immunofluorescence was detected in cultures of left or right side ganglia 6-8 days post incubation, or in cultures of dissociated ganglia explanted from normal uninfected mice.

Some dissociated DRG from uninfected mice were directly infected with ts 3 or wild type HSV-2 (at a multiplicity of 5 pfu per cell) and reincubated at 31°C. Cytopathic changes started to appear 12 hours post infection and progressed rapidly within the next few hours. Eighteen hours later, more than 50% of the cell population (infected with ts 3 at 31°C or with the wt virus at 38°C) were showing cpe (Fig. 19-a). At this time cells were fixed and labelled with fluorescence conjugated rabbit anti-HSV (Fig. 19-b). No difference was observed in the pattern of immunofluorescence in cell cultures infected in vitro with ts 3 or wt.

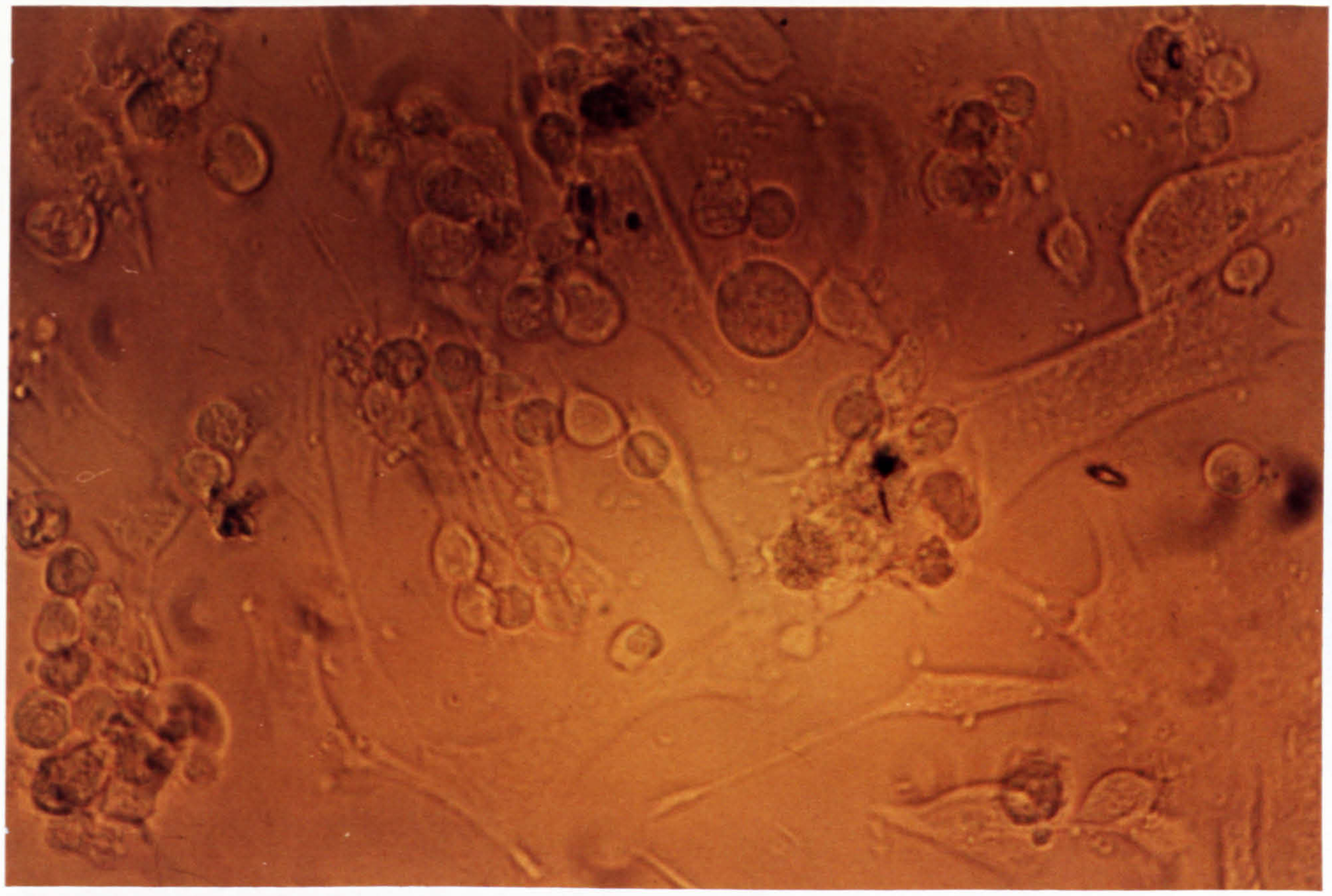
Cytopathic changes, due to virus reactivation, were noticed in HSV-2 infected dissociated DRG cultures on the 5th-6th day post dissociation (Fig. 20). However, virus was

FIGURE 19

a - cytopathic changes in cell culture of dissociated DRG infected with *ts* 3 virus (in vitro), 18 hours post infection at 31°C; magnification x320.

b - *ts* 3 infected ganglion cell showing positive, anti-HSV immunofluorescence, 18 hours after infection (*in vitro*); magnification x400.

a



b

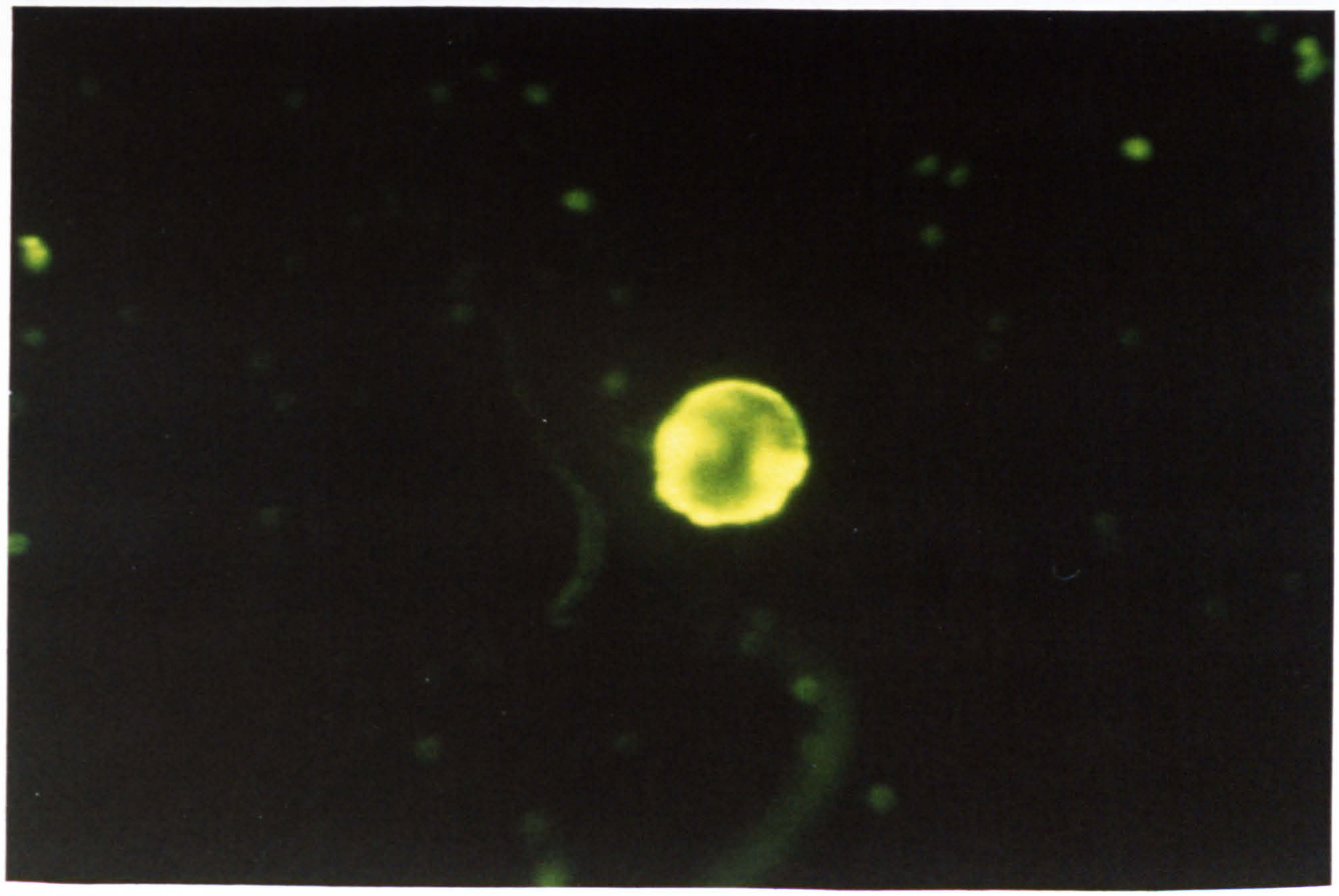
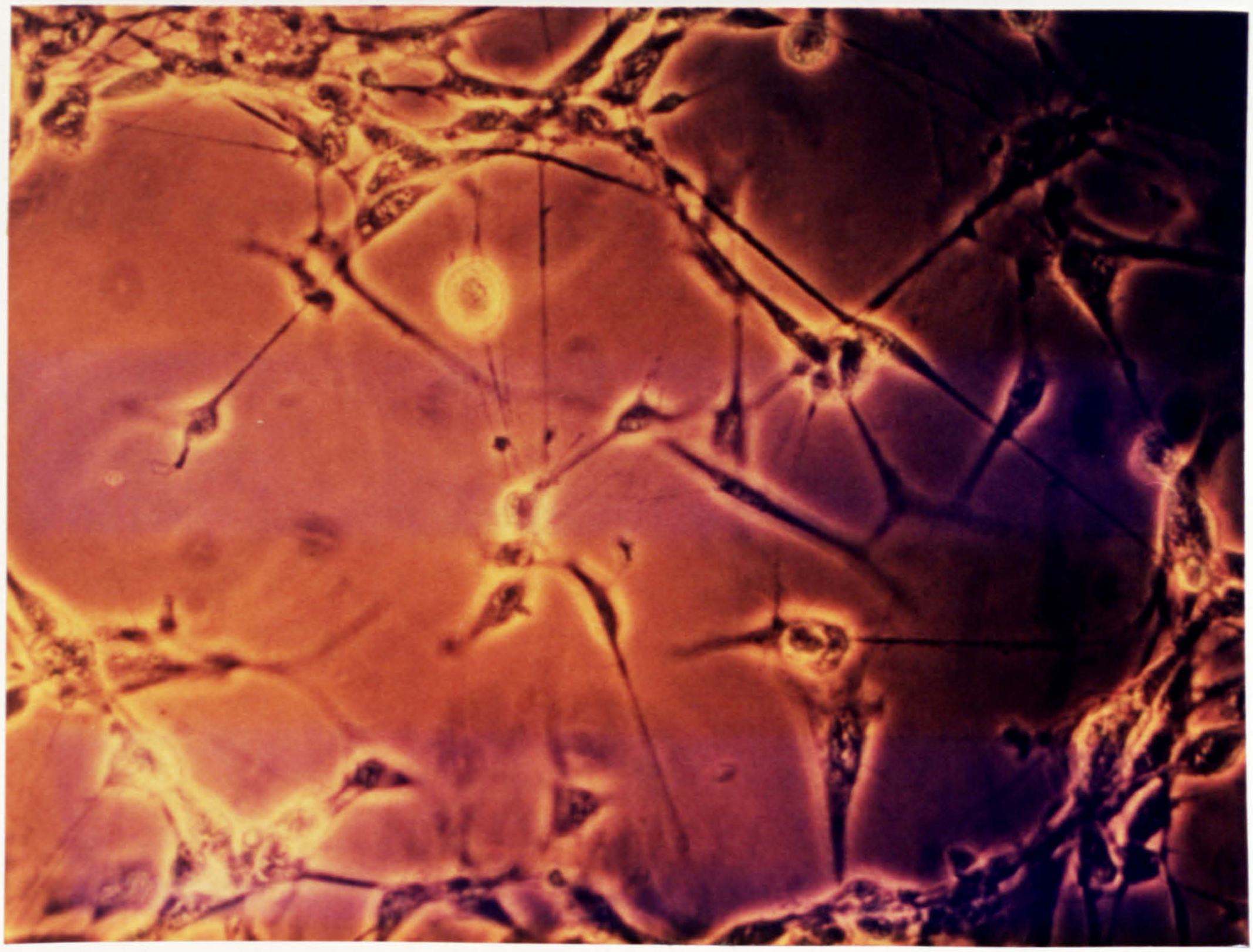


FIGURE 20

CYTOPATHIC CHANGES DUE TO REACTIVATION OF WILD TYPE VIRUS IN CELL CULTURE OF DISSOCIATED DRG (OF LATENTLY INFECTED MICE) 5 DAYS AFTER DISSOCIATION



Photographed with phase-contrast illumination showing the morphology and distribution of cells within the culture, magnification x320.

detected in the culture supernatant as early as the 3rd day after dissociation (see Table 30). The cpe areas were initially limited in distribution (1-2 foci per coverslip) but within the next 48 hours more than 40% of the cell population were showing cpe.

In some of the experiments (when the cpe was first noticed), the cultured cells were double-labelled with the neurone specific monoclonal antibody A_2B_5 and also with the poly-specific rabbit anti-HSV serum. All cells exhibiting a cpe were expressing viral antigen, and labelling of the cells with A_2B_5 showed that some of these cells were the neurones (Fig. 21a, b). A difference in the pattern of staining of cells with the two antibodies were observed. The DNA restriction enzyme profiles of the original inoculated virus (HG52) was compared to those of the recovered viruses. The DNA profiles were studied by using the restriction endonucleases Hind III, EcoRI, Kpn I and Bam HI.

The pattern of the DNA of the recovered virus was similar to that of the standard HSV-2 (HG52). However, a shift in the mobility of some of the restriction fragments (HindIII m, EcoRI m, Kpn r, s, BamHI g, u, z, a') was observed (Fig. 22). As discussed below experiments with ts mutants also revealed that some restriction fragments exhibited variable mobility.

7. Particle:pfu Ratio

In order to ascertain that the inoculated stock of virus does not have a large proportion of non-infectious particles, the wild type HSV-2 virus particles and that of ts 3, 5, 9 and 10 from the same stock used for mouse inoculation were counted using the electron microscopy

TABLE 30

VIRUS REACTIVATION IN DISSOCIATED DORSAL ROOT GANGLIA*

Mouse strain	Virus	Dose pfu/mouse	Virus detected in the supernatant at day**	Day cpe observed in neurones	No. of +ve culture/total
Pirbright	wt	10^6	5	6	3/6
Pirbright	wt	10^5	3	5	1/5
Pirbright	wt	10^5	4	6	1/5
Pirbright	wt	10^5	4	6	1/5
A	ts 3	1.2×10^5	-	-	0/4

* Ganglia from 4-5 mice were pooled for each experiment

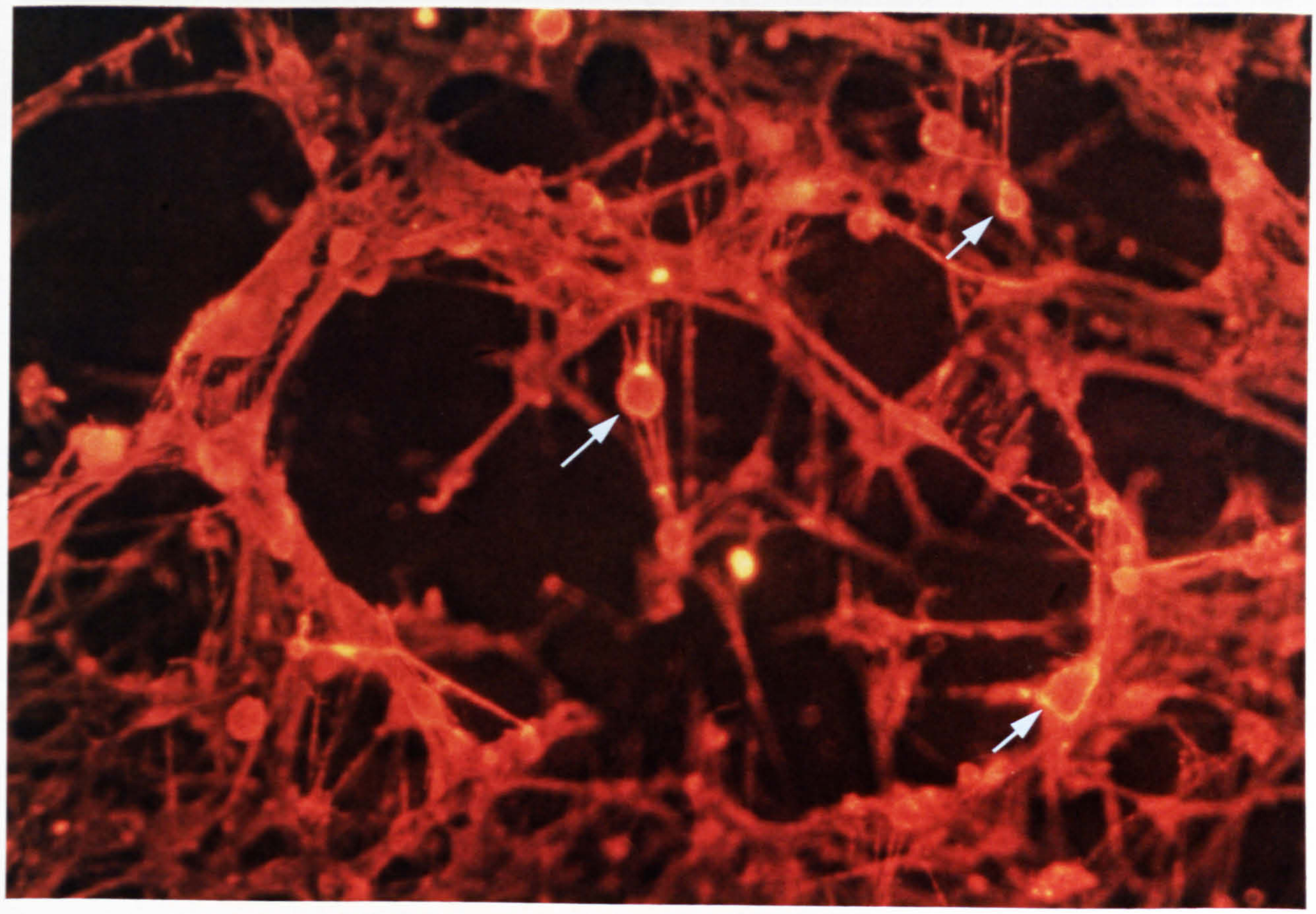
** Virus detected in the supernatant of dissociated DRG by plating 0.1 ml of the supernatant on BHK cells

FIGURE 21

CYTOPATHIC CHANGES DUE TO REACTIVATION OF WILD TYPE VIRUS IN CELL CULTURE OF DISSOCIATED DRG (OF LATENTLY INFECTED MICE) 5 DAYS AFTER DISSOCIATION

Same field as in Fig. 20 viewed under immunofluorescence microscopy; magnification x240. Note the positive rhodamin anti-HSV (a) and fluorescence A_2B_5 staining (b) in cells expressing viral antigen (large arrows). Also note the specific staining of some cells (expressing no viral antigen) with the A_2B_5 (small arrows).

a



b

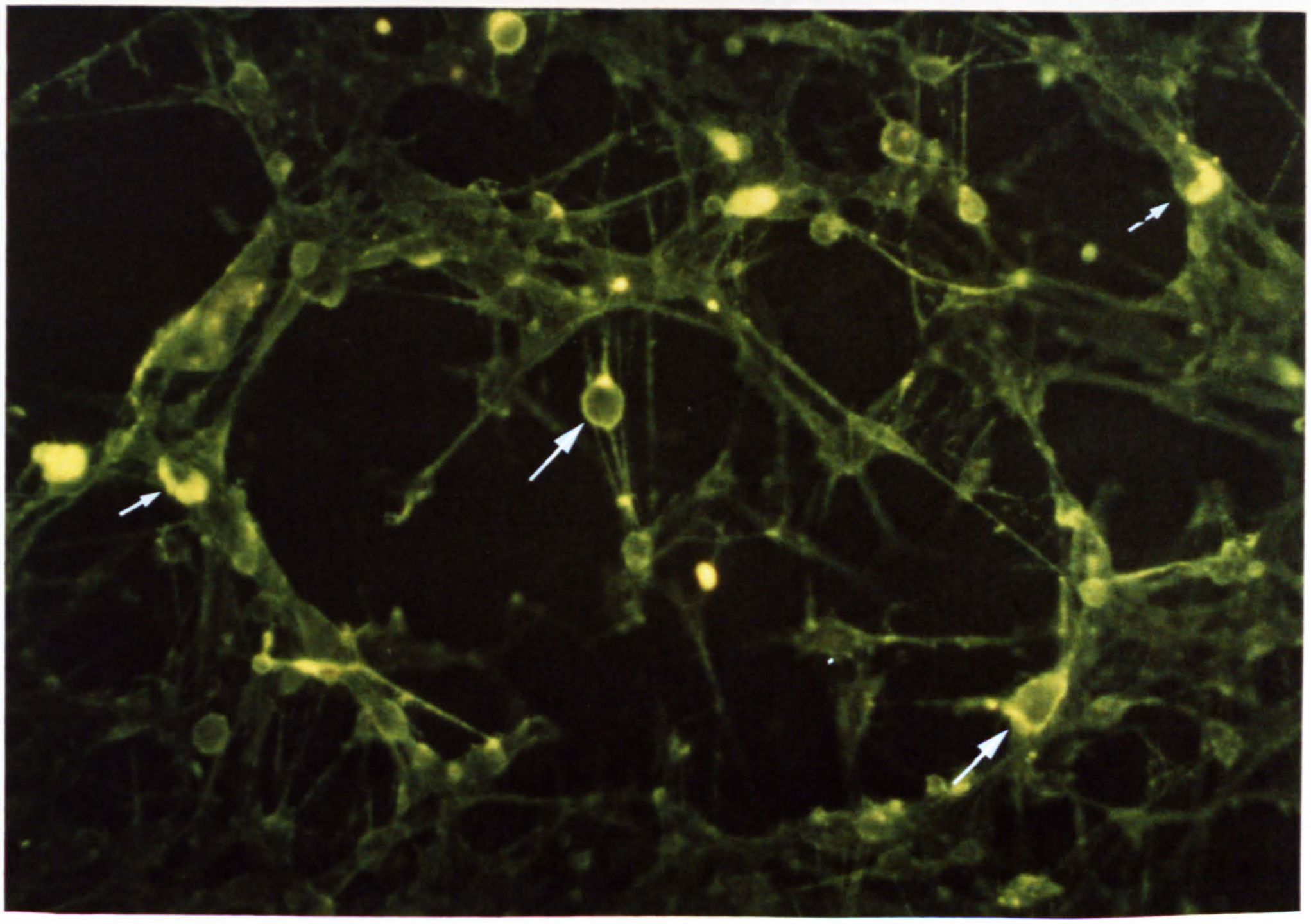


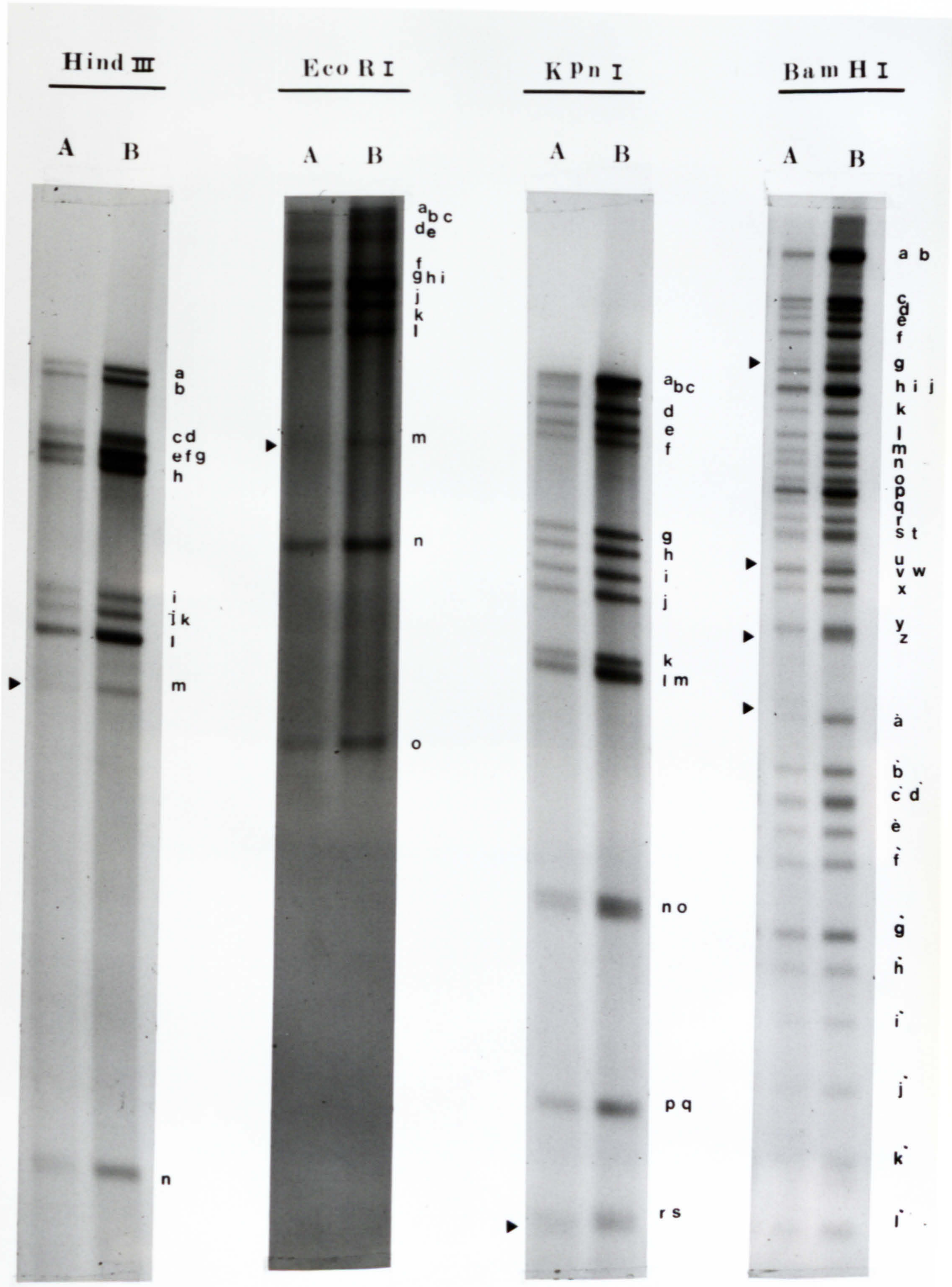
FIGURE 22

AUTORADIOGRAPHY OF HINDIII, EcoRI, KpnI AND BamHI RESTRICTION ENDONUCLEASE DIGESTS OF ^{32}P-LABELLED DNA OF HSV-2 (HG52) REACTIVATED FROM DISSOCIATED DORSAL ROOT GANGLIA

A latent-reactivated virus

B HSV-2 strain HG52

▶ restriction fragments showing variable mobility


Hind III
A B
a
b
c d
e f g
h
i
j k
l
m
n
Eco R I
A B
a b c
d e
f
g h i
j
k
l
m
n
o
Kpn I
A B
a b c
d
e
f
g
h
i
j
k
l m
n o
p q
r s
Bam H I
A B
a b
c
d
e
f
g
h i j
k
l
m
n
o
p
q
r
s t
u
v w
x
y
z
à
b̀
c̀ d̀
è
f̀
ġ
ḥ
ì
j̀
k̀
l̀

negative staining technique.

The particle to pfu ratios of these virus stocks were found to be within the acceptable limit (633, 102, 48, 54 and 110 respectively).

8. Growth of Wild Type HSV-2 and Some of the ts Mutants in Mouse Brain

To determine whether ts 3, ts 5, ts 9 (which were very deficient in establishing latent infection in sensory ganglia) and ts 10 grow in the mouse brain, these viruses were inoculated (at a dose of 10^3 pfu/0.05 ml) directly into the brains of female BALB/C mice. After a given time interval the mice were killed then their whole brains were individually removed, homogenised, sonicated and later titrated both at the permissive and non permissive temperatures. The growth curves of these mutants and that of the wild type were then estimated and compared (fig. 23).

The results show that inoculated viruses start to disappear in the first 30 minutes after inoculation and were no longer demonstrable in the mouse brain 2 hours after infection. The wild type HSV-2 virus started to reappear 12 hours later and continued to increase in titre attaining the maximum level 3-5 days post infection; but the ts mutants could not be demonstrated in the mouse brain following the initial 2 hours of infection. Some wild type virus presence in the brain tissue was still detectable as late as 12 days post infection.

9. Titration of HSV-2 Wild Type and ts Mutants at Different Temperatures of Incubation

In order to ascertain whether the ts mutants produced plaques at temperature other than the (31°C) permissive temperature, the mutants and the wild type virus were

FIGURE 23

GROWTH CURVES OF THE WT AND TS MUTANTS VIRUSES IN THE MOUSE BRAIN

Two or more mice were used at each time interval.

● wt

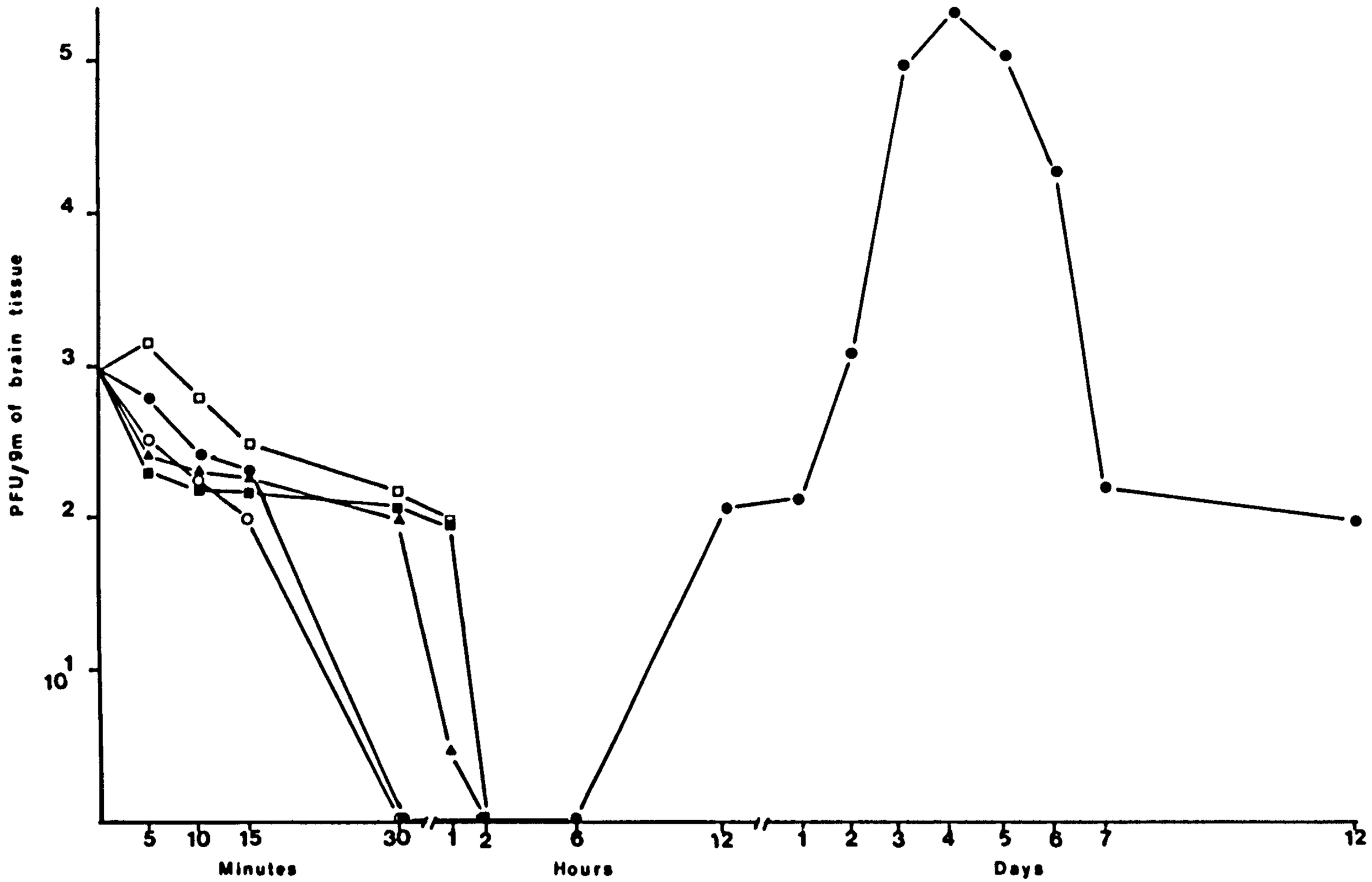
■ ts 3

▲ ts 5

□ ts 9

○ ts 10

The experiment for the mutants and wt viruses were carried out for 12 days post inoculation.


PFU/9m of brain tissue
5
4
3
2
10^1
5
10
15
30
1
2
6
12
1
2
3
4
5
6
7
12
Minutes
Hours
Days

titrated in BHK cells at four different temperatures (31°C, 34.5°C, 36.5°C and 38.5°C) of incubation.

The result of such experiments (Fig. 24) indicated that none of the ts mutants produced plaques at the nonpermissive temperature (38.5°c). All the mutants, however, produced similar plaque yields at the other three temperatures of incubation; except that progressive drop in ts 9 plaque formation was observed at 34.5 and 36.5°C temperature of incubation.

10. Thermostability of HSV-2 wt and ts Mutants Under Explanted Ganglia Conditions

In order to determine the thermostability of the wt virus and the ts mutants at 31°C, the virus stocks (3 ml of virus suspension in EFC 50%) in screw capped vials were incubated at this temperature for up to seven days. Stocks were sampled and titrated at 31°C at a chosen interval of time. Results are given in Fig. 25.

The pH of the incubated virus stocks were not estimated, however, a slight change in the colour of viral suspension was noticed in the later days of the incubation. At this temperature of incubation all the ts mutants progressively lost titre, 1,000 fold (3 logs) being the minimum in seven days (ts 1). Most mutants (ts 2, 3, 4, 6, 7, 10 and 12) survived seven days incubation losing 4-5 logs of titre, while ts 5, 8, 9, 11, 13 and the wt viruses were all essentially lost by day 7. The titre of ts 5, 11 and 13 appeared to drop more rapidly from the second day post incubation than did the titre of most other mutants. The results of these experiments provides evidence that the method used for screening ganglia (2-3 times/week) and footpads (daily) should have allowed the efficient recovery

FIGURE 24

TITRATION OF WT AND TS MUTANT STOCKS AT FOUR DIFFERENT TEMPERATURES OF INCUBATION: 31°C, 34.5°C, 36.5°C & 38.5°C

None of the mutants showed any detectable plaque formation at 38.5°C.

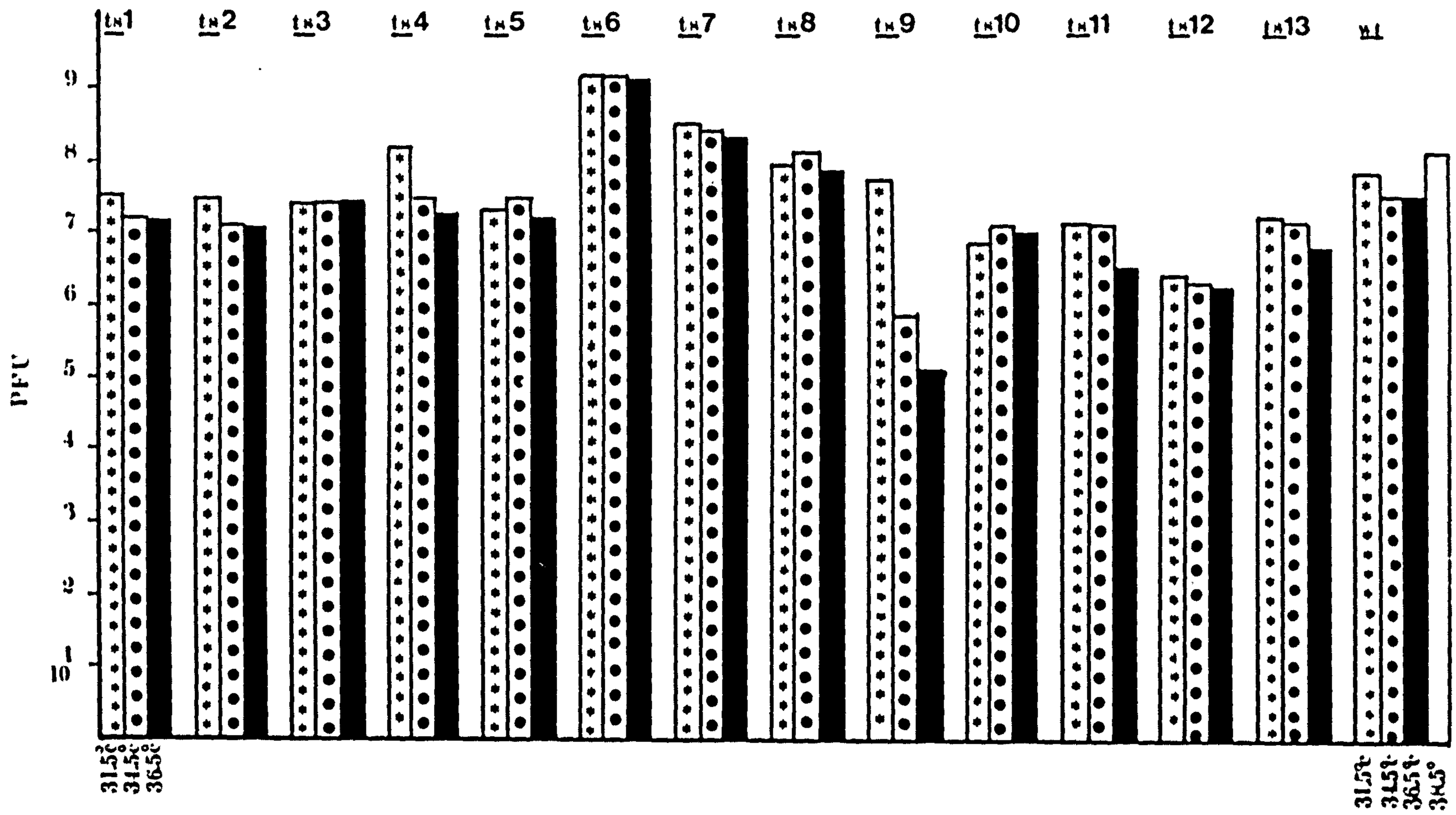
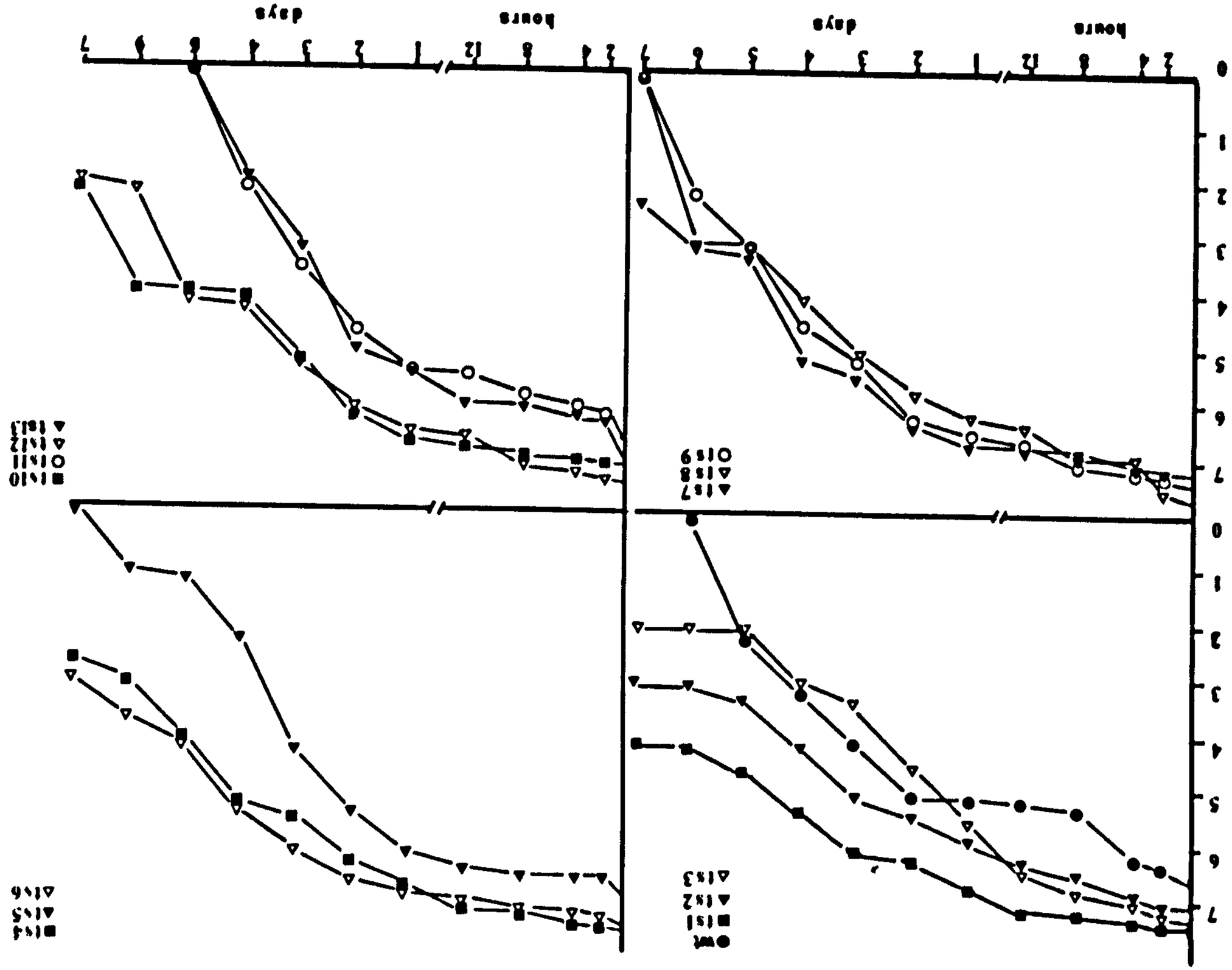

tn1
tn2
tn3
tn4
tn5
tn6
tn7
tn8
tn9
tn10
tn11
tn12
tn13
wt
PFU
0
1
2
3
4
5
6
7
8
9
315°
345°
365°
315°
345°
365°
345°

FIGURE 25

THERMOSTABILITY OF HSV-2 (WT) AND 13 TS MUTANTS AT 31°C


wt
1s1
1s2
1s3
1s4
1s5
1s6
1s7
1s8
1s9
1s10
1s11
1s12
1s13
hours
days
hours
days

of released viruses. In addition, all the recovered viruses were infectious, growing in BHK cells and hence replication of reactivated viruses in explanted ganglia or footpad tissue can be expected at the temperature of incubation (31°C).

11. Homogenisation of Footpad Tissues

In order to check for the presence of infectious wt virus in the footpad tissues of latently infected mice at the time of explantation, the individually explanted footpad tissue, from BALB/C mice, was divided into two halves. One half was homogenised and titrated while the other half was kept in organ culture and screened for released virus. The sensory ganglia of all of the six latently infected mice shed virus in their supernatants.

Tissues from acutely infected and from uninfected BALB/C mice were also homogenised and titrated as positive and negative controls respectively.

The wt virus could not be demonstrated in any of the homogenised halves of footpad tissue while 4/6 cultured halves released wt virus after 8-12 days of incubation (Table 31). No virus was recovered from the homogenised or cultured tissues explanted from uninoculated left hind limb.

No loss of virus has been observed to take place during the process of homogenisation of footpad tissues explanted from acutely infected mice or when the virus was added to already explanted footpad tissue which was then homogenised (Table 32).

The results of these experiments showed that the virus can be recovered from the footpad tissue, in a fashion similar to its recovery from the latent state in the sensory

TABLE 31

COMPARISON OF WT VIRUS BEHAVIOUR IN HOMOGENISED AND NON-HOMOGENISED HALVES OF FOOTPAD TISSUES FROM LATENTLY INFECTED MICE*

Mouse No.	Footpad tissue		Day at which the virus was detected in the supernatant
	Homogenised half	Non-homogenised half**	
1	-	+	8
2	-	-	-
3	-	+	12
4	-	+	8
5	-	+	11
6	-	-	-

* BALB/c mice were initially inoculated with (HG52) 10^6 pfu/mouse into the footpad

** Supernatant were screened daily at 38°C

TABLE 32

EFFECT OF THE HOMOGENISATION PROCESS ON THE TITRE OF WT VIRUS INOCULATED IN THE FOOTPAD TISSUE BEFORE OR AFTER EXPLANTATION

Titre of input virus (pfu)	No. of footpads used and status	Titre after homogenisation
10^5	1 already explanted	1.6×10^5
10^4	1 already explanted	4.4×10^4
10^2	1 already explanted	3.2×10^2
2×10^5	2 acutely infected	2.5×10^5
2×10^4	2 acutely infected	1×10^4
2×10^2	2 acutely infected	1.5×10^2

ganglia i.e. only after several days of incubation and not by homogenisation.

12. Temperature-Sensitivity of Reactivated Viruses

Viruses reisolated after reactivation from mice which had been initially infected with ts mutants were checked for possible reversion. All recovered viruses proved to have retained their ts genotype; all grew at 31°C but not at 38°C.

13. Analysis of Restriction Endonuclease Digest of the DNA of Recovered Viruses

Stocks of reactivated viruses were prepared and their DNA restriction enzyme profiles were compared with that of the respective virus initially used for mouse inoculation. ^{32}P-labelled DNA was prepared from individual recovered viruses and digested with BamHI, BglII, HindIII and EcoRI. DNA fragments were then separated on agarose gels which were dried and autoradiographed. Results are shown in Fig. 26a-e. All recovered viruses were identified as HSV-2 by comparison with the standard HG52 strain of HSV-2. Restriction fragments BamHI g, p, u, z, a'; EcoRI m and HindIII m exhibited variable mobility. These fragments contain sequences from the terminal repeat/inverted repeat regions of the genome (see restriction endonuclease map, Fig. 27) and have previously been recognised as having variable mobility (Chaney et al., 1983). Mobility variation was also observed in BamHI w and y which map in U_L (Fig. 26 a and b). Loss of the HindIII m-1 restriction site was observed in a single case of ts 3 virus recovered from the footpad (Fig. 26d). This will be discussed later.

FIGURE 26 (a-e)

AUTORADIOGRAPHS OF THE RESTRICTION ENDONUCLEASE DIGESTS OF ^{32}P-LABELLED DNA OF TS MUTANTS VIRUSES REACTIVATED FROM GANGLIA AND FOOTPADS EXPLANTS

Figure

a BamHI restriction endonuclease digests

b BamHI restriction endonuclease digests

c EcoRI restriction endonuclease digests

d HindIII restriction endonuclease digests

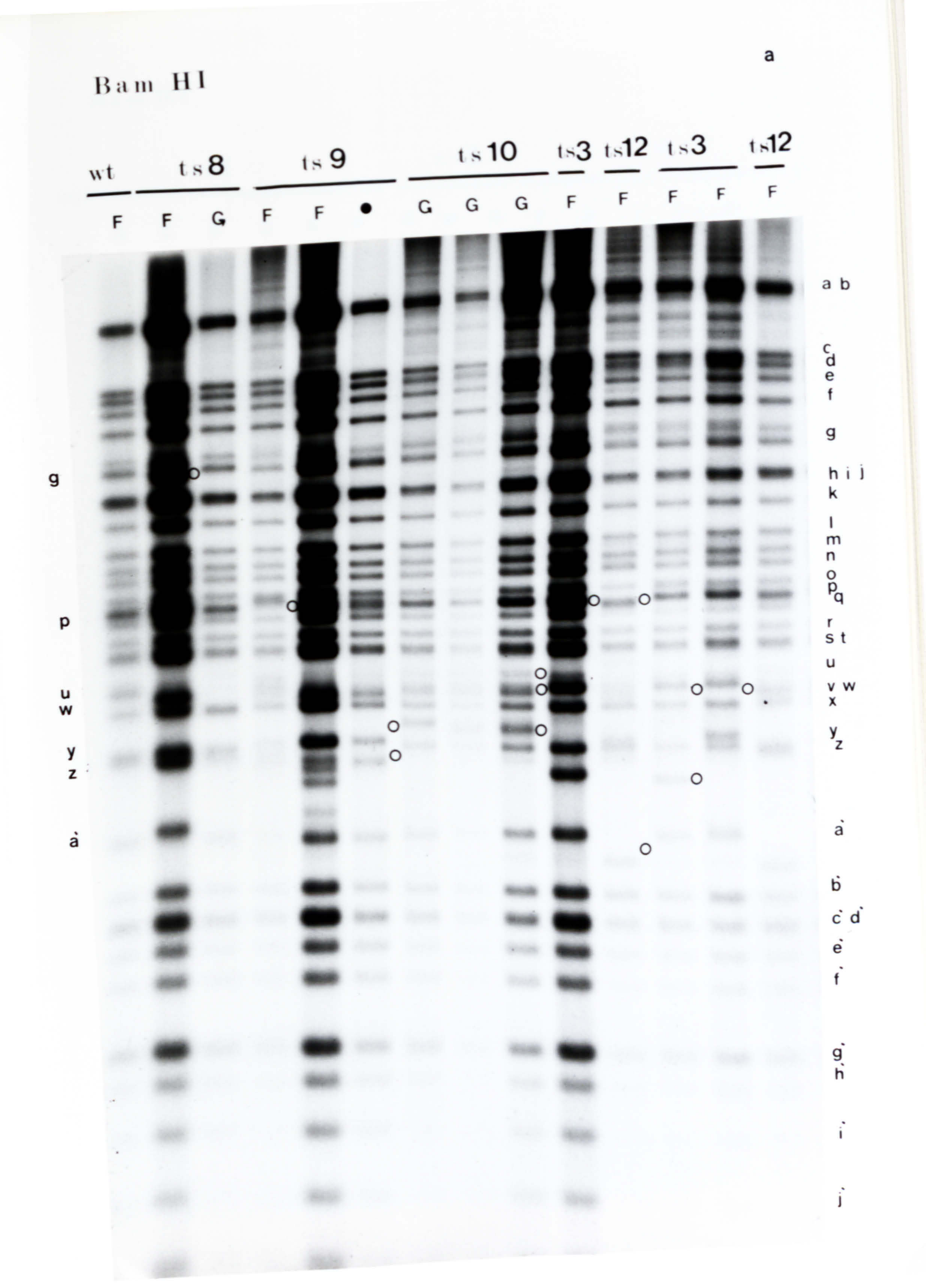
e BglII restriction endonuclease digests

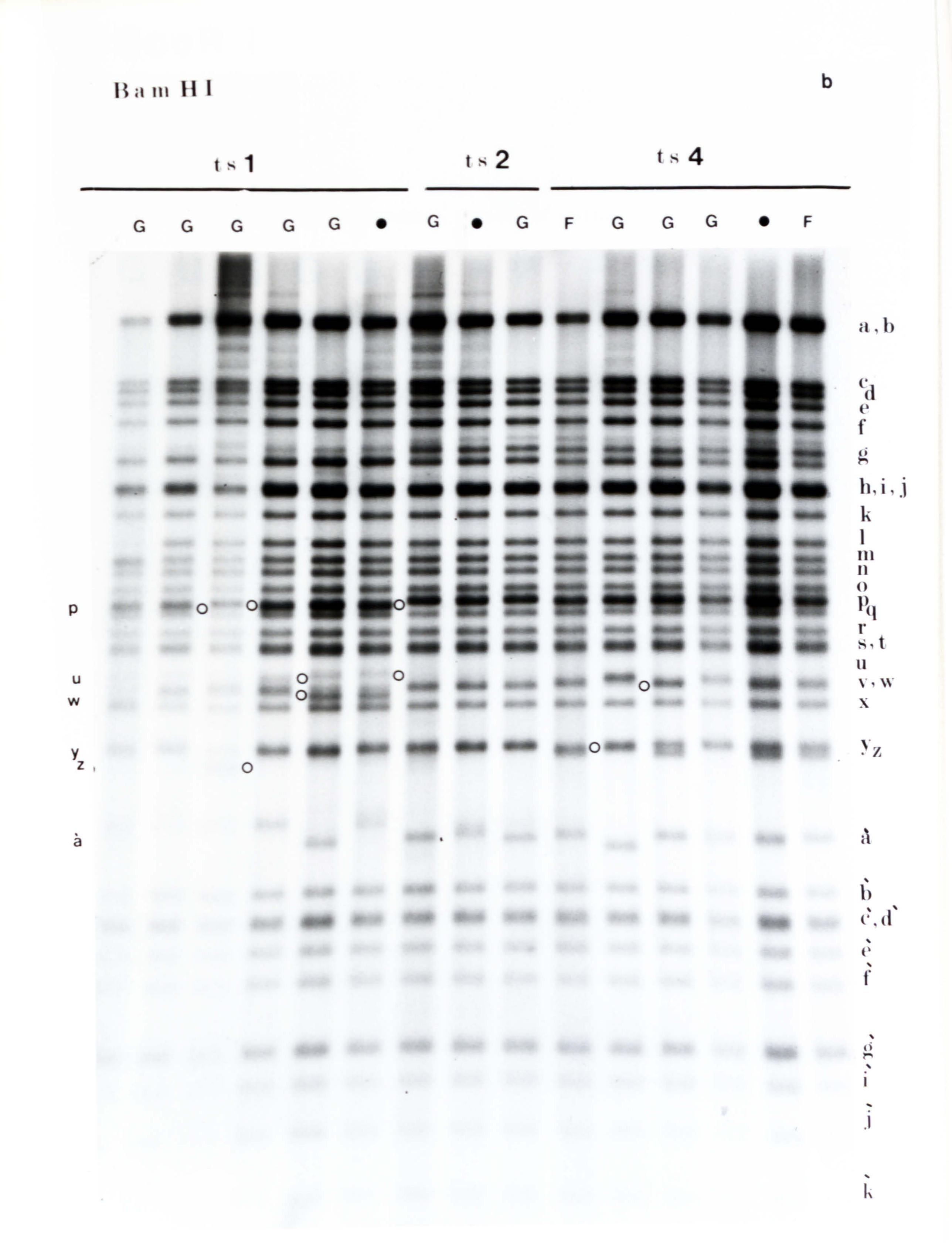
Restriction fragments with variable mobility are indicated with the symbol (o)

F virus recovered from the footpad

G virus recovered from the ganglia

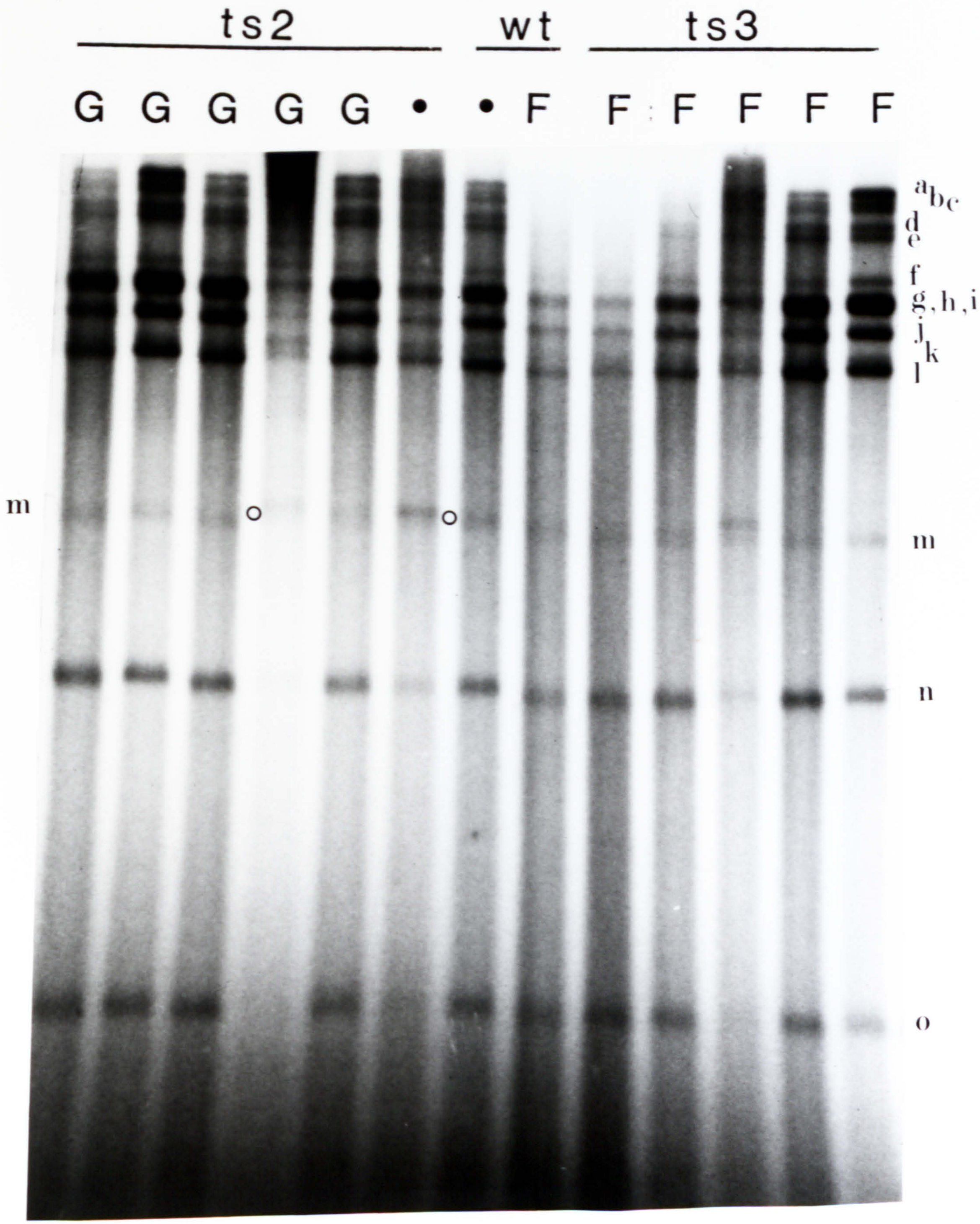
● initially inoculated viruses


a
Bam HI
wt
ts8
ts9
ts10
ts3
ts12
ts3
ts12
F F G F F ● G G G F F F F F
a b
c
d
e
f
g
h i j
k
l
m
n
o
p q
r
s t
u
v w
x
y
z
a'
b'
c' d'
e'
f'
g'
h'
i'
j'
g
p
u
w
y
z
a'


BamHI
b
ts 1
ts 2
ts 4
G G G G G ● G ● G F G G G ● F
a, b
c
d
e
f
g
h, i, j
k
l
m
n
o
p
q
r
s, t
u
v, w
x
y
z
à
b̀
c̀, d̀
è
f̀
p
u
w
y
z
à

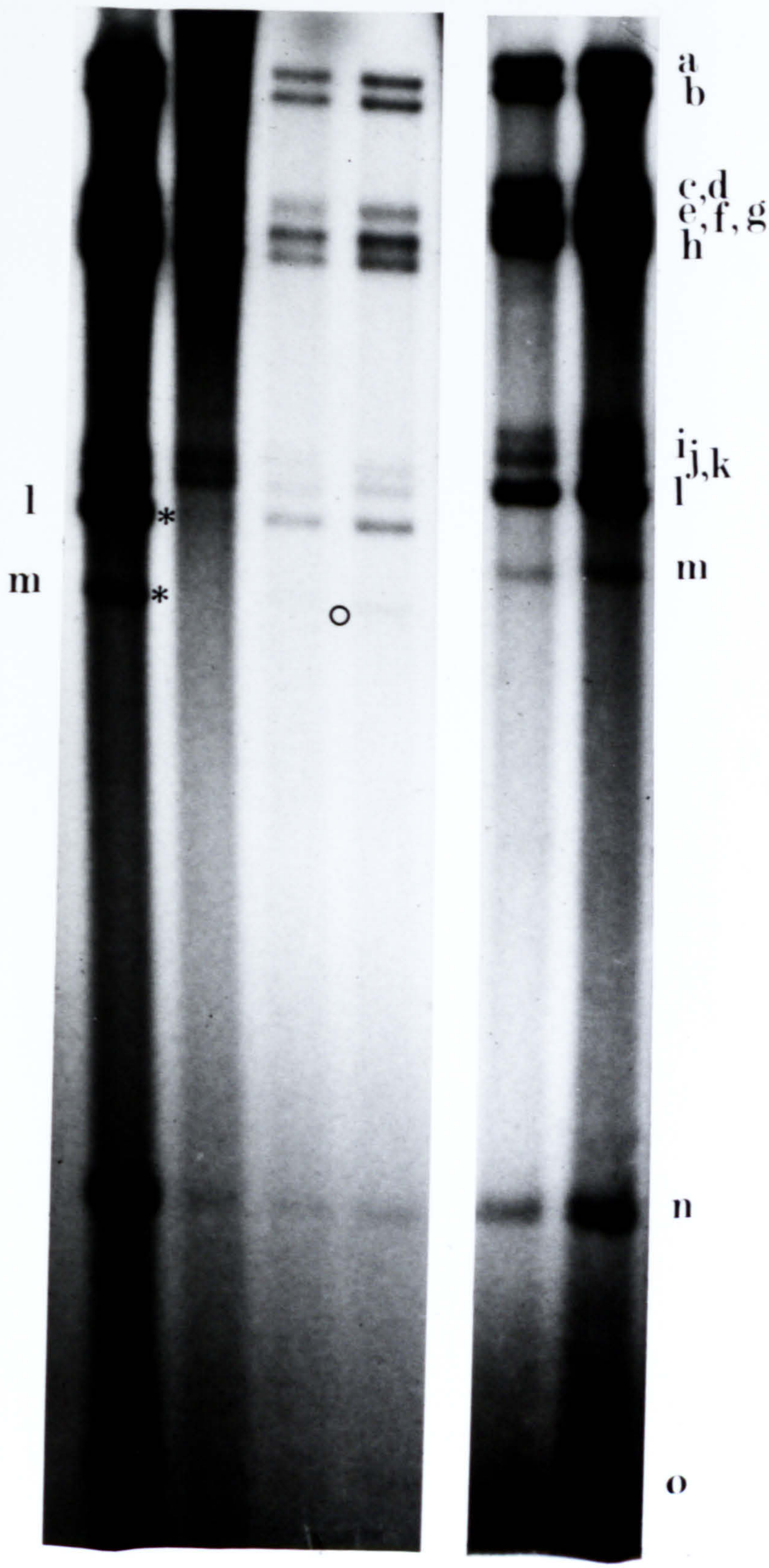
EcoR I

c



Hind III

d



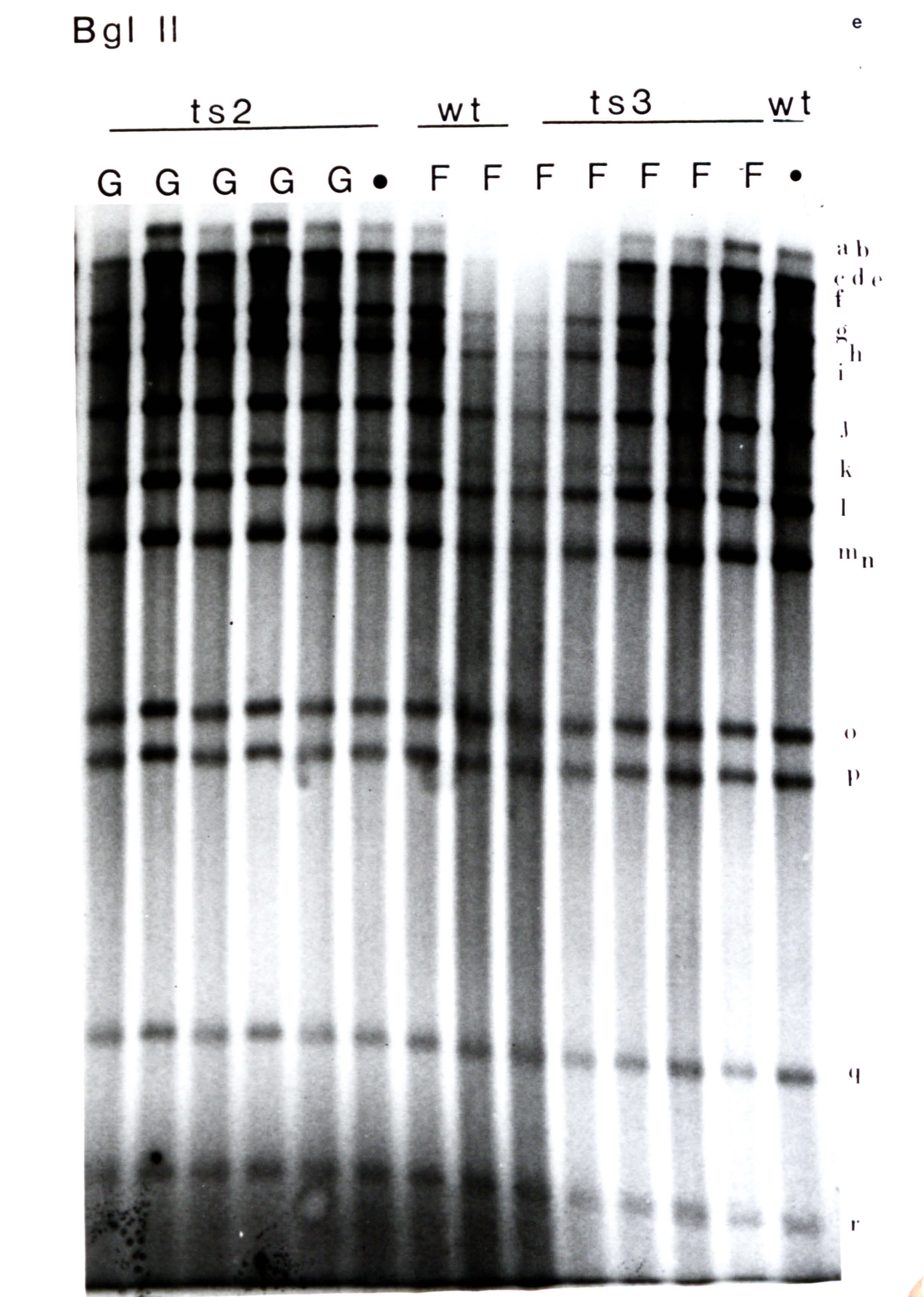

Bgl II
ts2
wt
ts3
wt
G G G G G • F F F F F F F •
a b
c d e
f
g
h
i
j
k
l
m
n
o
p
q
r

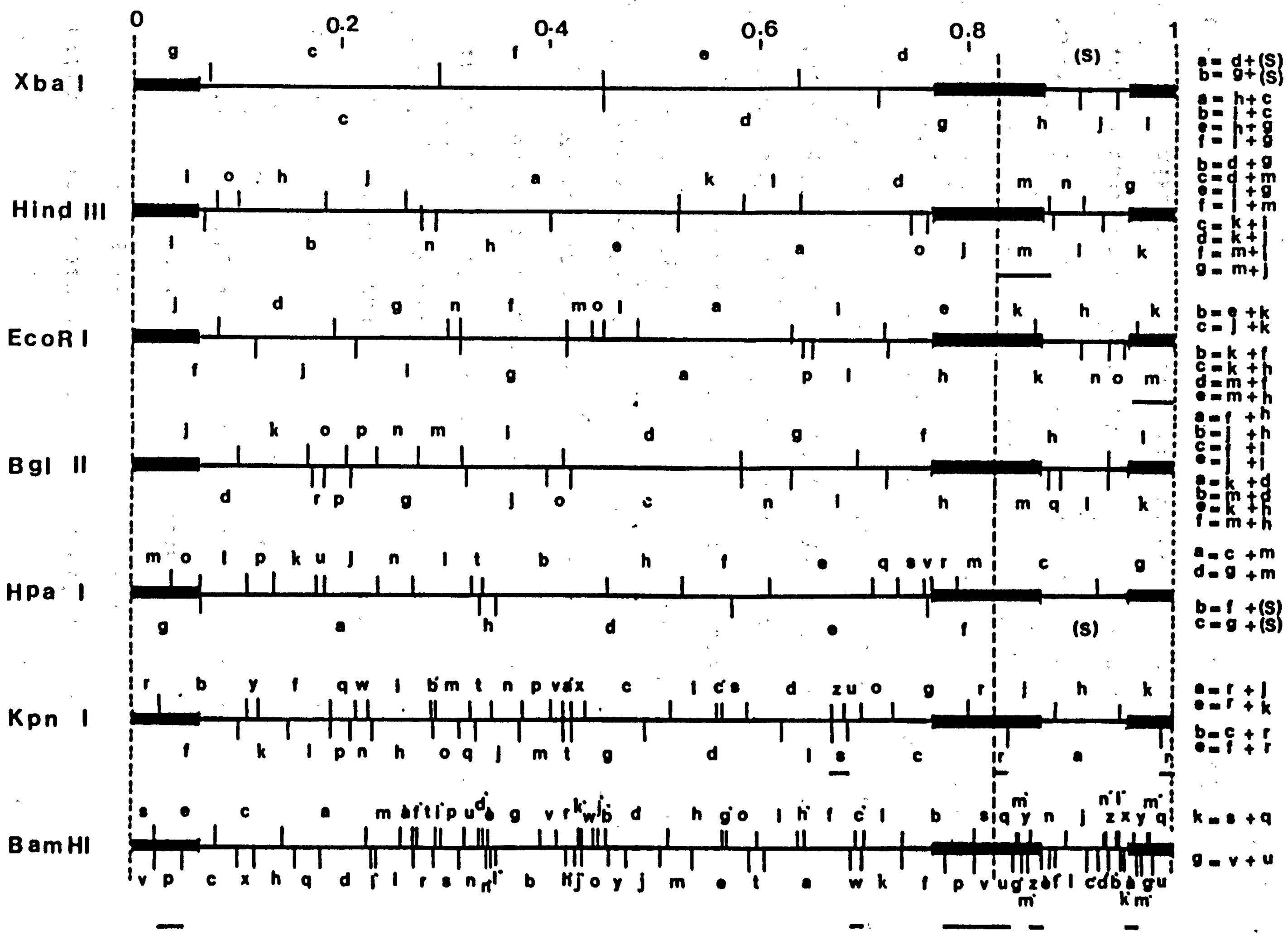
FIGURE 27

RESTRICTION ENDONUCLEASES PHYSICAL MAPS OF HSV-1 (STRAIN GLASGOW 17) AND HSV-2 (STRAIN HG52) DNAS

Letters above the genome model refer to HSV-1 DNA fragments while those of HSV-2 are shown below the genome model.

The composition of the joint fragments is given beside the maps. Only the prototype arrangement of U_L and U_S is shown (after Davison, 1981).

Lines below the genome model refer to the fragments with variable mobility.


0
0.2
0.4
0.6
0.8
1
Xba I
Hind III
EcoR I
Bgl II
Hpa I
Kpn I
BamHI
(S)
a = d + (S)
b = g + (S)
b = e + k
c = j + k
b = c + r
e = f + r
k = s + q
g = v + u

14. Histologic Studies of Explanted DRG

The aim of this particular study was to determine the survival time of ganglion cells after explantation and incubation at 31°C.

Explanted ganglia from uninfected mice were formalin fixed, paraffin sectioned and stained with haematoxylin & eosin stain after culturing for various times post explantation. The slides of sectioned ganglia were studied under light microscopy (Fig. 28).

Healthy ganglion cells (neurones) have been observed in sections of ganglia that were fixed and stained immediately after explantation (zero time). The cells appear as rounded bodies of large and small sizes. Their nuclei were large with prominent nucleoli. Neurones were separated from the surrounding connective tissue by flattened (satellite) cells. Some shrinking neurones were also observed in this section, this could be due to the traumatising effect of the explantation and/or fixation-staining procedure. The number of healthy neurones decreases in sections of ganglia that have been incubated for a longer period. Pathological changes and cell degeneration start to appear on the fourth day post incubation and progress rapidly with the subsequent days. A complete degeneration of the neurones was observed in sections of ganglia that have been incubated for 10 days or more post explantation. However, other supporting cells, satellite and connective tissue cells, have retained a normal looking morphology even after incubation for up to 3 or 4 weeks.

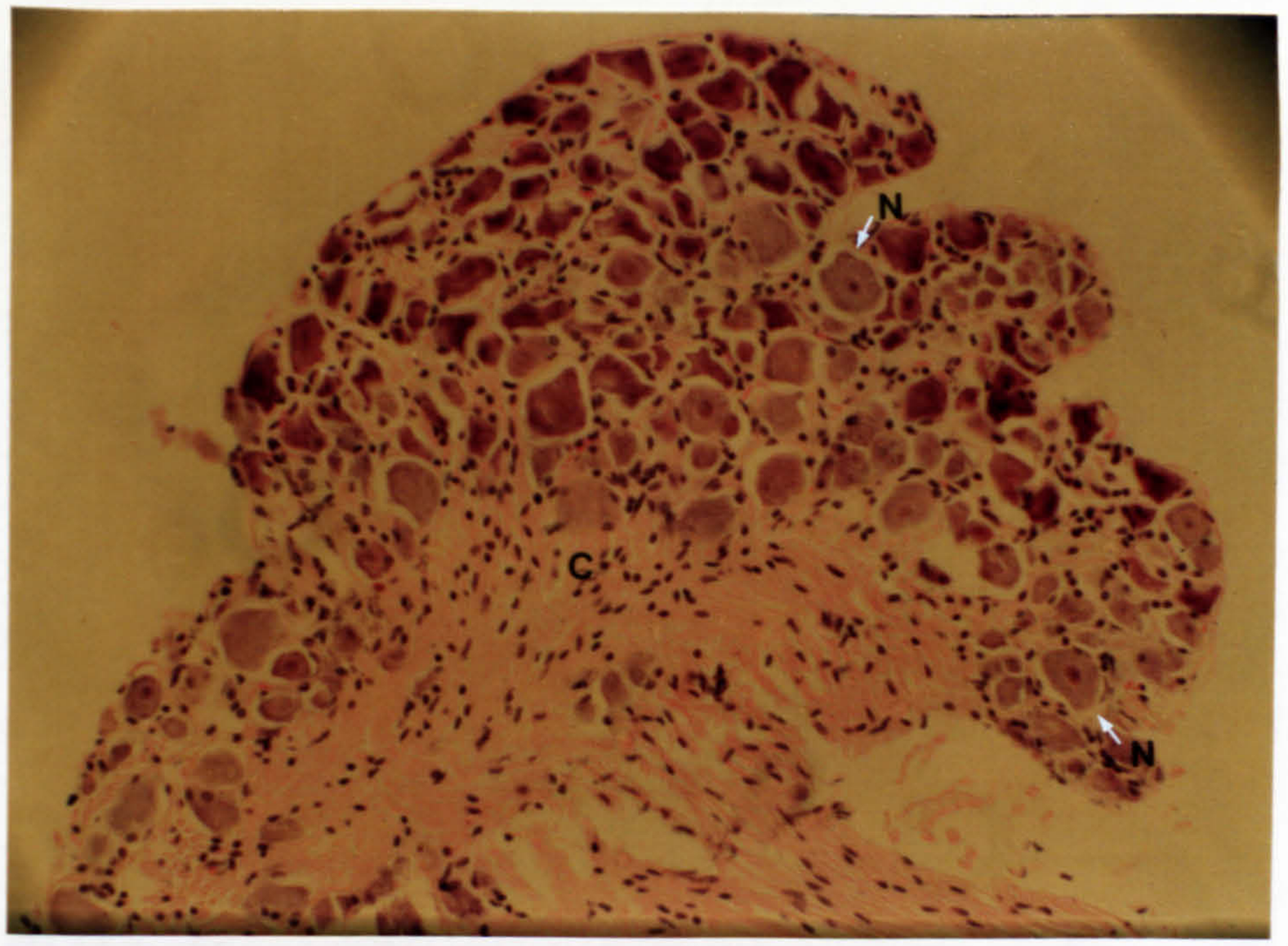
FIGURE 28

PHOTOMICROGRAPHS OF GANGLION CELLS AS THEY APPEAR IN SECTIONS STAINED WITH HEMATOXYLIN AND EOSIN

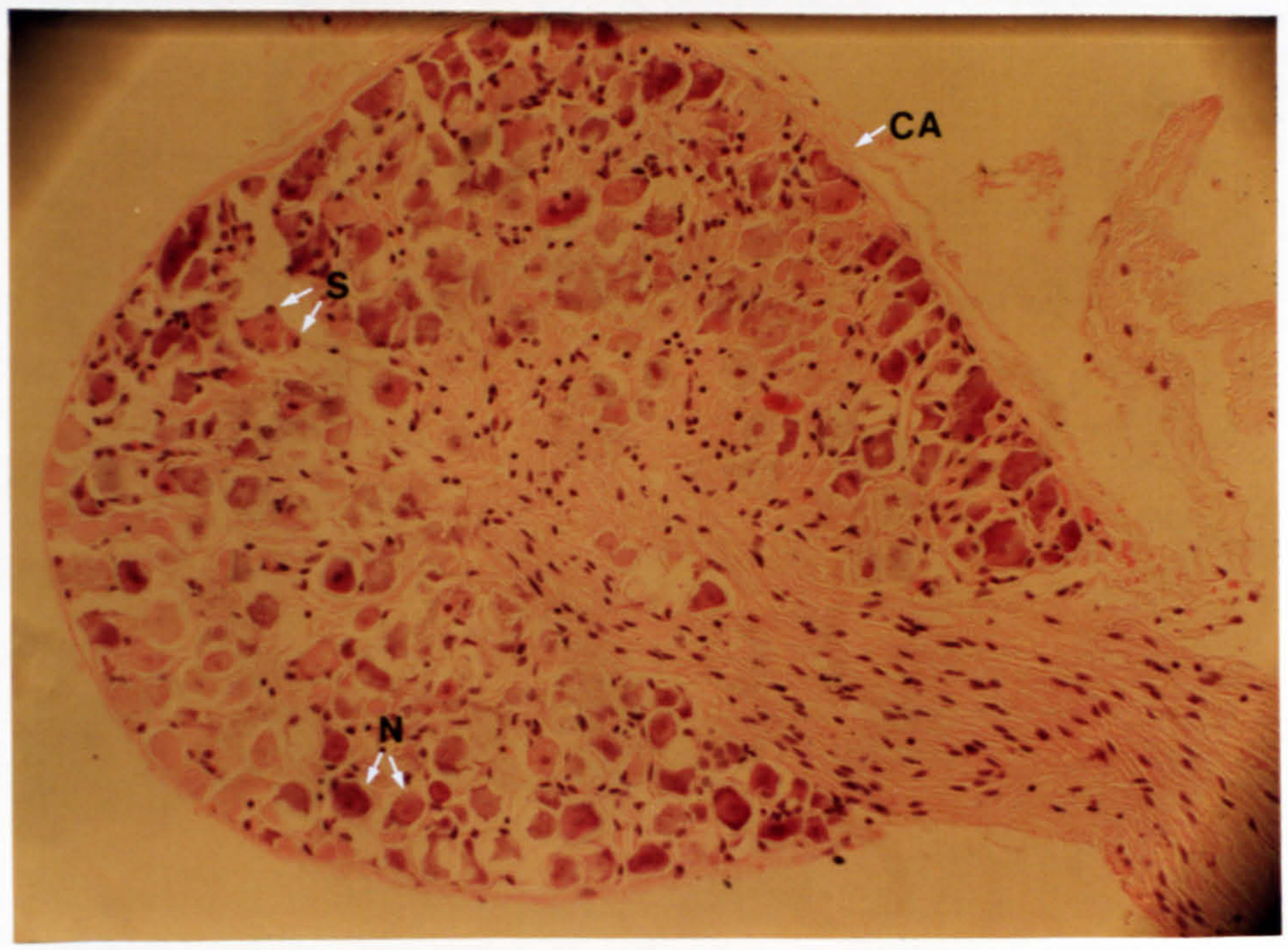
Mouse sensory ganglia were explanted and incubated as described. Note the healthy looking neurones (both large and small size) in the first two photomicrographs, where the explanted ganglia were fixed and stained immediately after explantation (a), or 2 days after being in culture (b); magnification x250 and x200 respectively. Pathological changes in ganglion neurones were first noticed on day four post incubation (c) and progress rapidly with the subsequent days (6, 8, 10 days) of incubation (d, e, f), magnification x250. However, a substantial number of neurones have retained the normal morphology. Almost complete degeneration of ganglion cells (except the connective tissue cells) was observed in ganglia incubated for 2 and 4 weeks post explantation (g, h); magnification x200.

N	neurone
S	satellite cell
CA	capsule
C	connective tissue
P	pathological changes

a



b



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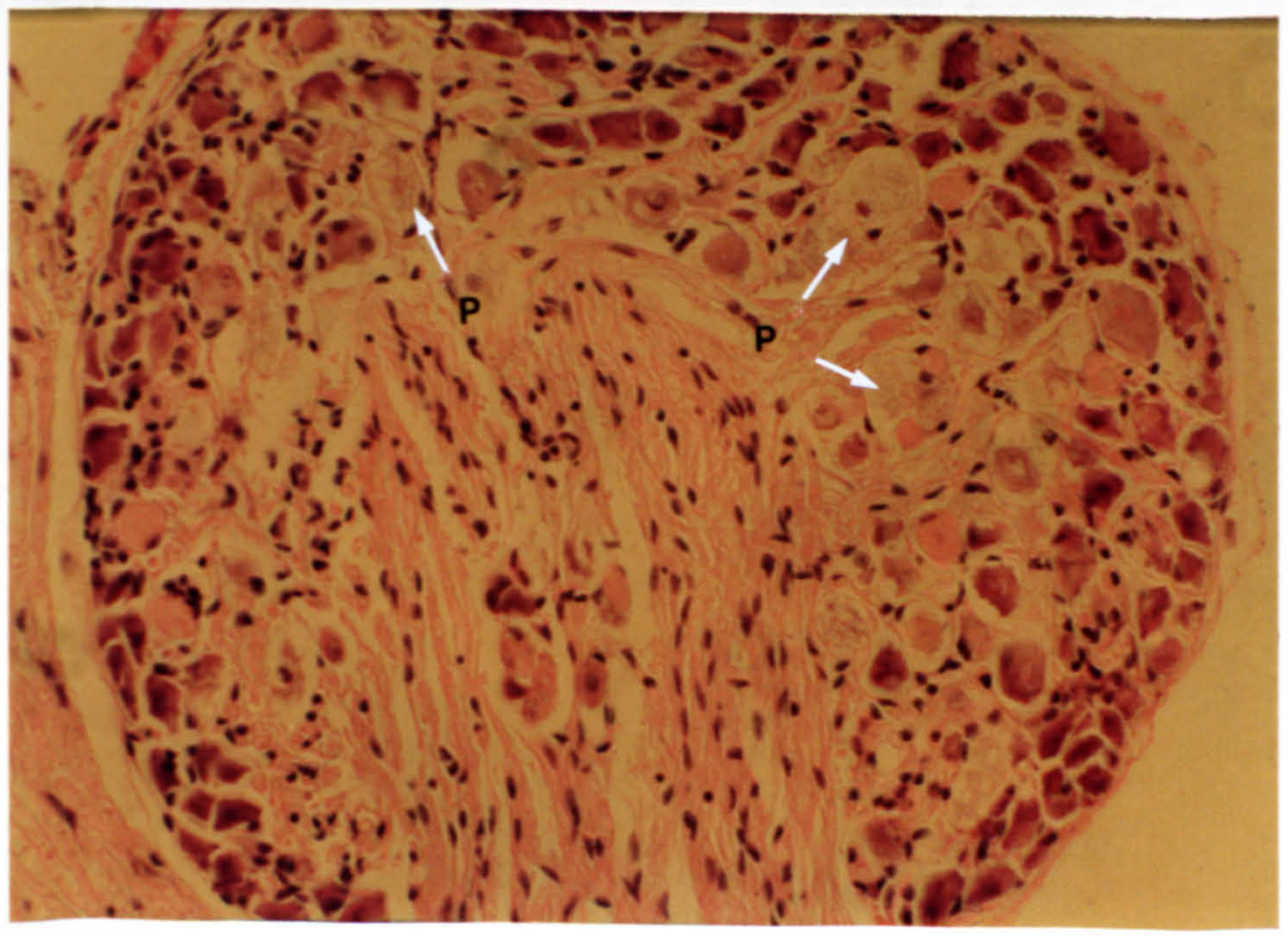

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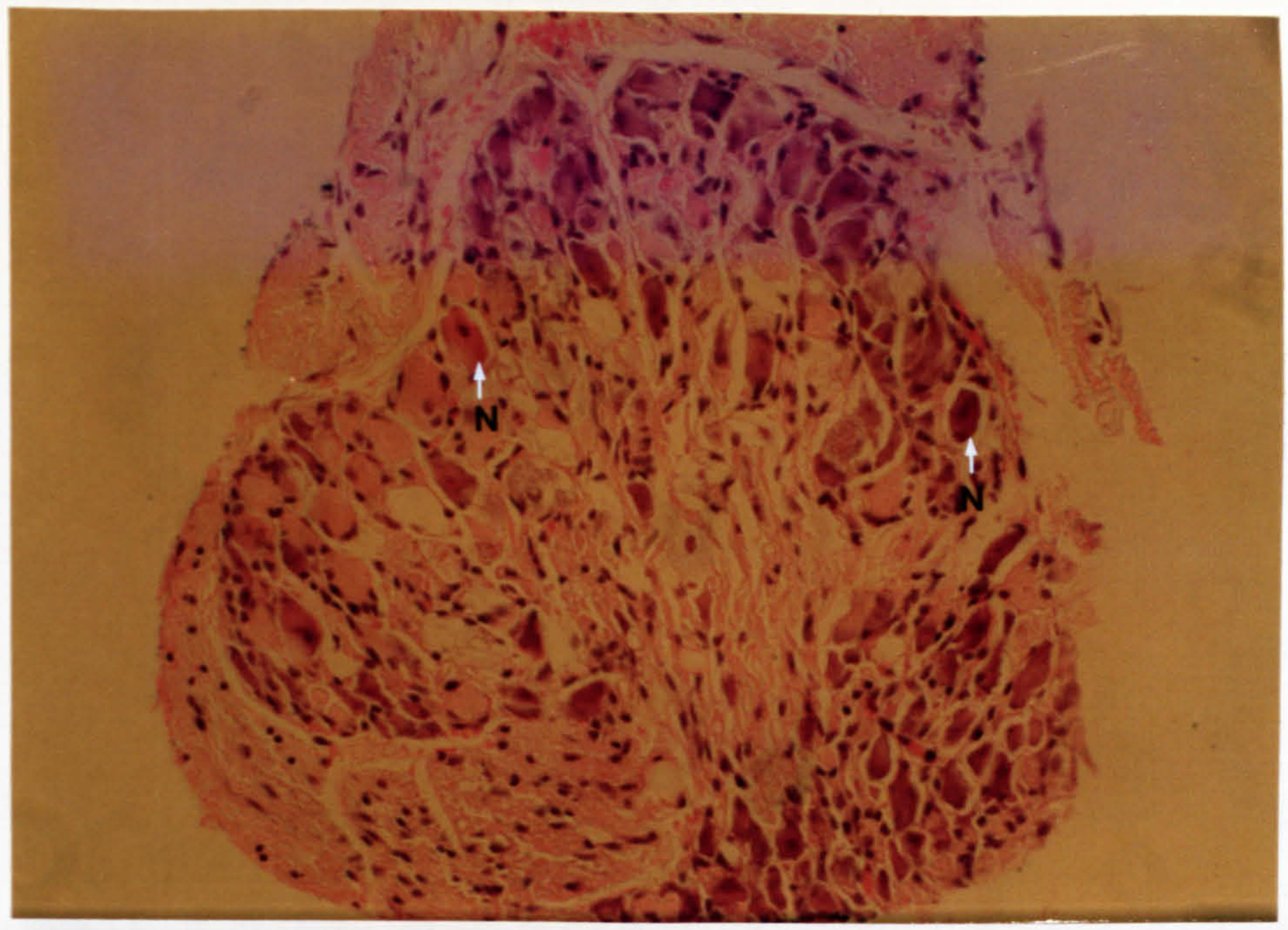

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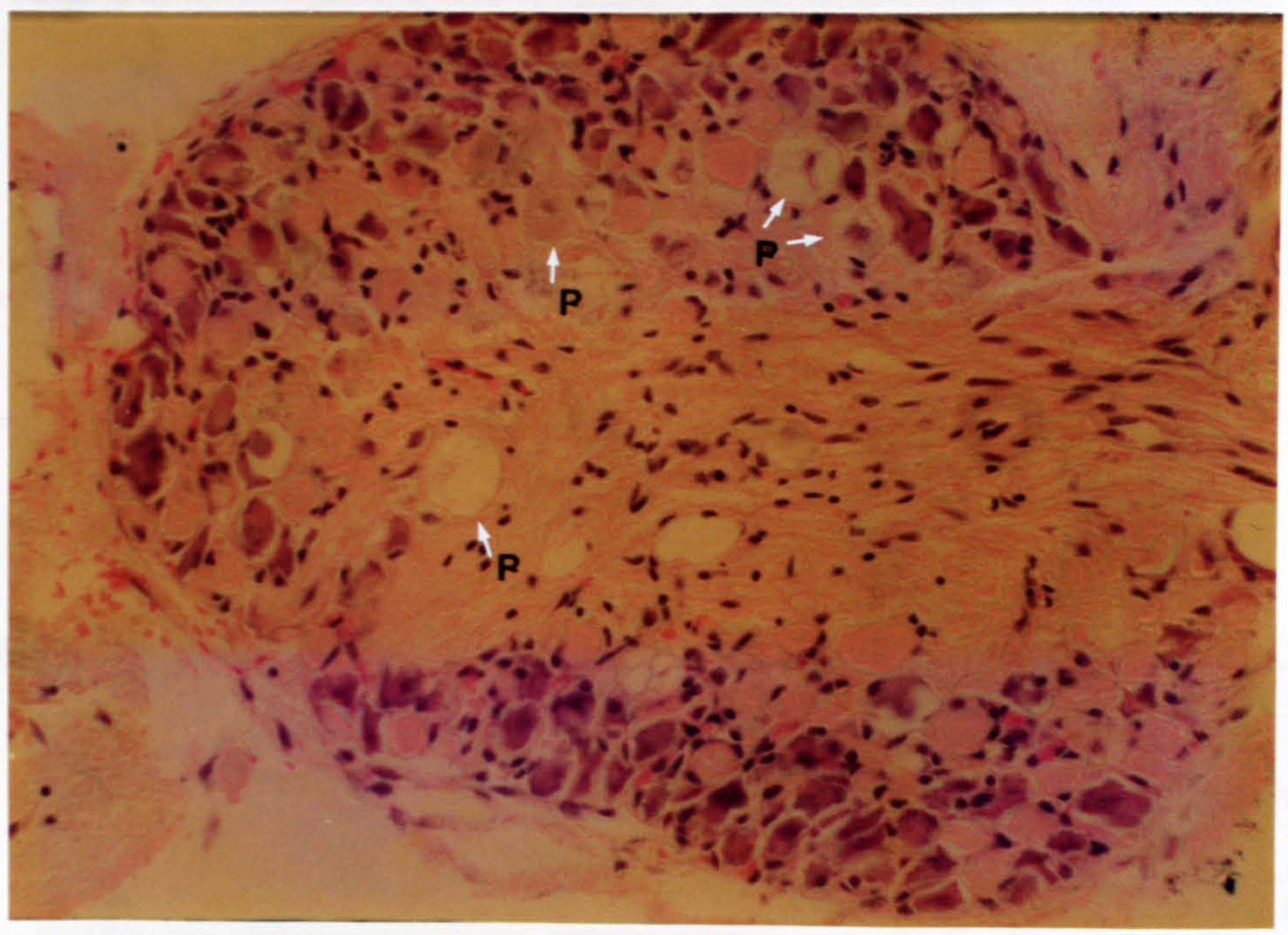

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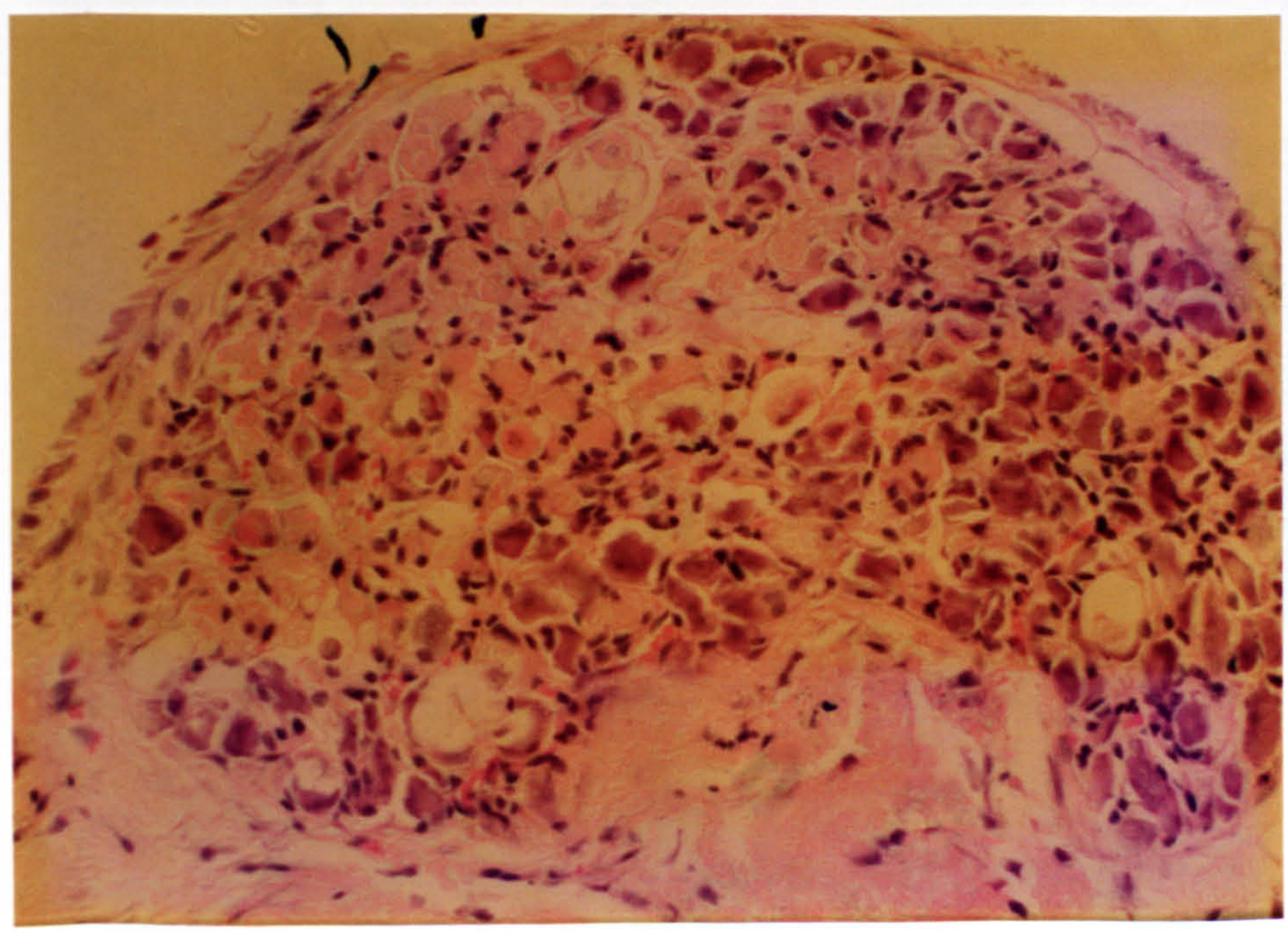

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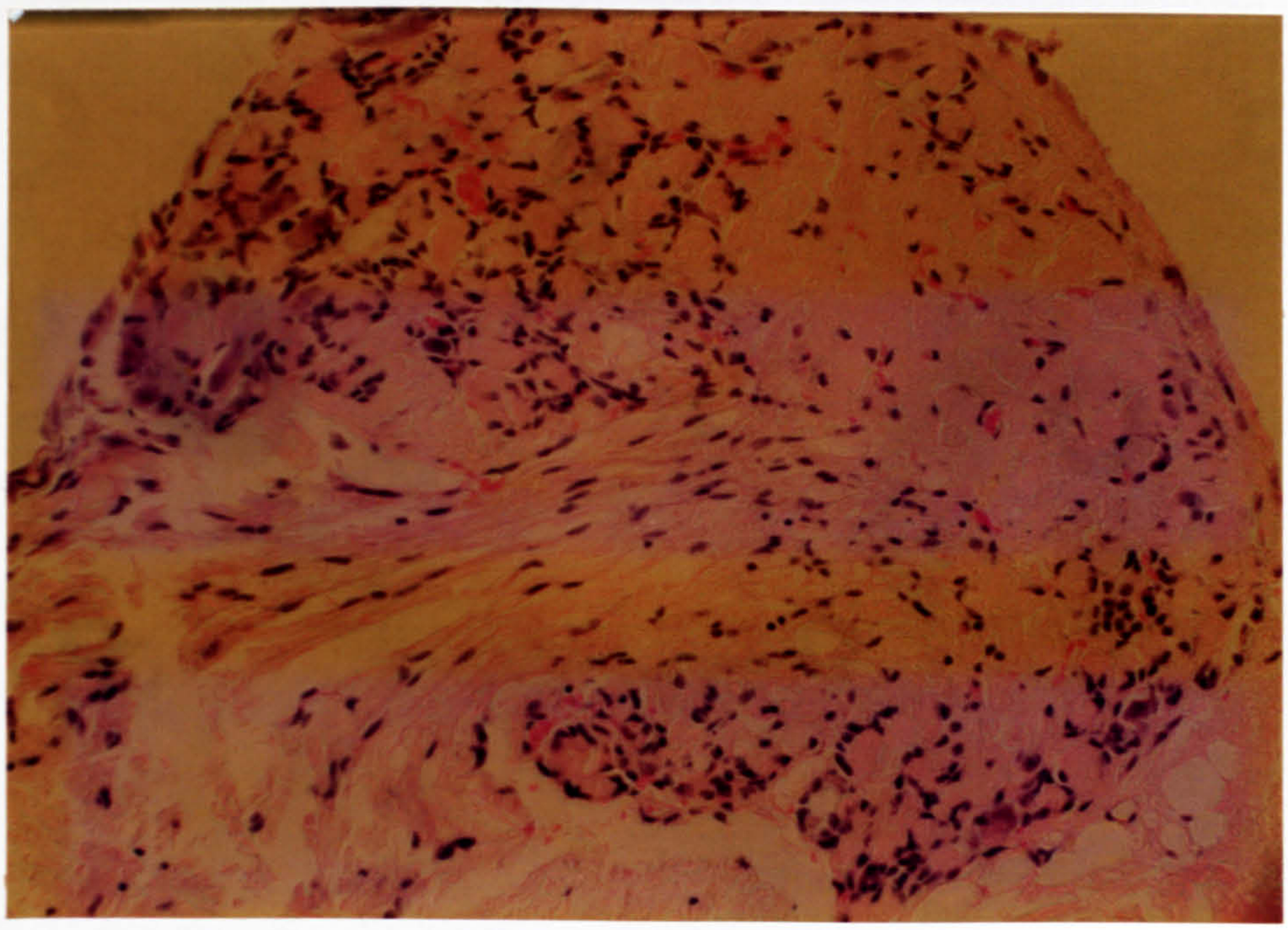

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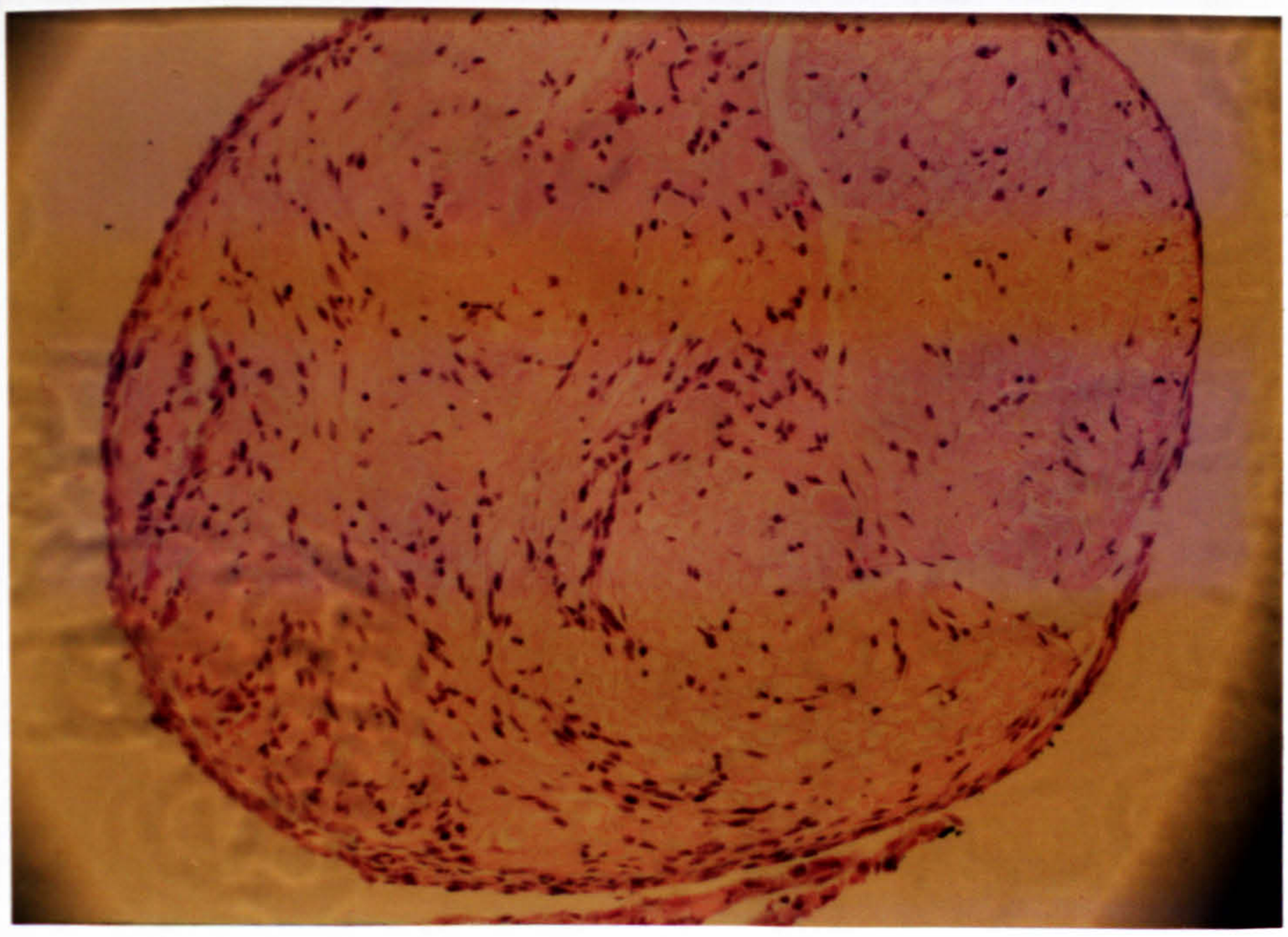

DISCUSSION

For a very long time, Herpesviruses have been known to cause latent infection; in fact the ability of the fever blister (cold sore) causative agent to establish latent infection has been postulated (Goodpasture, 1929) in the early years of this century.

Herpes simplex virus is of two distinct serotypes, HSV-1 and HSV-2, and both are known to produce recurrent herpes. Following a primary infection in man, herpes simplex virus usually persists in the latent state and remains there life long. Man may be infected with both types 1 and 2 and have recurrences caused by either or both (Wheeler, 1975). The site of recurrence appears to be determined by the location of the primary infection (Change, 1971) and the viral genome appears to be latent within the sensory neurones of dorsal root ganglia (McLennan and Darby, 1980). Though the virus has been isolated from other sites within the body for example from the trigeminal nerve root (Warren et al., 1982), cervical tumour cells grown in culture (Aurelian et al., 1971) and from the uterosacral ligament (Eglin et al., 1981); it is not clear whether the virus is truly latent in these tissues or has reactivated from the corresponding ganglia and subsequently travelled to these sites. The possible existence of latent virus in peripheral tissue has not been excluded. The physical state of the viral genome during the latent phase of infection is unknown and neither viral genes involved in latent infection in man nor host factors that may determine the susceptibility or resistance of man to the latent infection have been identified.

The aim of the work presented in this thesis was to identify and to study viral genes that may influence the ability of HSV to induce latency in the mouse. It is axiomatic

that such genes can be characterized through the use of HSV temperature-sensitive mutants that may be deficient in establishing latent infection. The possible occurrence of variation in the (genetically determined) host's defense system of resistance both to the acute and latent HSV infection can also be studied through the observation of virus behaviour in different host strains.

Recovery of wt Virus From the Latent State

After virus inoculation (see Methods), mice were observed for signs of acute infection and then survivors were left for (at least) another 3 months before they were sacrificed and the presence of reactivable virus in their explanted tissues scored.

The results of the experiments presented in this study show that strain HG52 of HSV-2 (wt) behaves differently in individual mouse strains which differ in their resistance both to the acute and latent infections. As seen from the data of Table 6 mouse susceptibility to acute HSV-2 infection is influenced partly by the dose of infection and partly by the host. The resistance of BIOZZI mice to the acute infection at both tested doses was high. However, since the virus was not inoculated at doses higher than 10^6 pfu, the effect of still larger doses of virus has not been investigated. Published studies with inbred strains of mice infected with HSV-1 showed that mouse strains are either resistant to 10^6 pfu C57BL/6-J, moderately susceptible, BALB/c, or very susceptible A-J (Lopez, 1975; Kirchner et al., 1978a). The results in Table 6 confirmed the high susceptibility of "A"/mice to HSV infection by extending observations to HSV-2. This susceptibility is shown not only by the criterion of the number of mice which died after infection (7/12 at 10^6 pfu), but in addition by the

fact that paralysis or death was also noticed in mice that were inoculated with lower doses of virus, and the observation of a shorter incubation period in this particular strain of mice. However, the results with HSV-2 are not completely consistent with the categorization of the inbred strains, C57BL/6-J and BALB/c as resistant and susceptible respectively (Lopez, 1975). In my experiments (Table 6) BALB/c strain mice were resistant to moderately susceptible. However, it is important to note that i, small numbers of BALB/c mice were used in this particular experiment, ii, different routes of inoculation were used in the two studies (i.e. intraperitoneal compared to footpad inoculation), iii, there is a difference in virulence between the two serotypes (HSV-1 and HSV-2). Although, HSV-1 and HSV-2 are very similar they are far from identical and it is noteworthy that there is much evidence of genetic divergence between the genomes of the two serotypes (Halliburton, 1972; Kieff et al., 1972; Ludwig et al., 1972; Cortini and Wilkie, 1978). The two serotypes differ in many aspects of their biological properties including the nature of the infections they induce in their natural host (Nahmias and Roizman, 1973a, b, c). Furthermore, it has been reported that HSV-2 is more neurovirulent in mice than HSV-1 when inoculated either intramuscularly or into the footpad, however, there were no differences in virulence upon intracerebral inoculation or when a high dose of inoculum was used (McKendall, 1980).

Based on the experiments reported here (Table 6) mouse strains can be categorized (judged by the total percentage of mortality) as resistant (BIOZZI, BALB/c, C57BL/6-J), moderately susceptible (PIRBRIGHT) and very susceptible (A/mice) when inoculated with 10^5 pfu. However, when mice were inoculated with 10^6 pfu the categorization will be slightly different, for

now C57BL/6-J, BALB/c and the PIRBRIGHT mice all appeared to be moderately susceptible. This effect of dose on the classification scheme reinforces the importance of precisely defining the infectious doses used when ascertaining comparative resistance or susceptiblity of mouse strains and it underlines the conclusion that resistance is not an "all or none" phenomenon (Lopez, 1981).

It is well known that macrophages (mononuclear phagocyte system) can respond to viral infection in a specific (immune) and non specific (non immune) fashion, and that the latter function of macrophages plays an important role in interfering with and in limiting virus dissemination within the host in the early stages of primary infection (Mogensen, 1979). It is of interest to know that A/J mice (very susceptible to HSV infection) were found to have a profound defect in macrophage (tumour cytotoxicity) function (Borraschi and Meltzer, 1980), and it follows that macrophages might play a role in the increased susceptiblity of A/mice to intraperitoneal infection with HSV-1 (Lopez, 1981). However, at the present time no information is available about the cytotoxicity function of macrophages in other strains of mice and analysis of interaction between HSV-2 and macrophages (derived from susceptible and resistant mice) is relevant and important. Although the exact mechanism of virus restriction by macrophages is not known, it is probable that macrophages induce their effect through the release of virus-destructive products, probably lysosomal enzymes, and/or by processing and presenting captured antigens to lymphocytes for immune recognition (Unanue, 1972). Further, macrophages have been shown to be of importance in the production (by specifically sensitized lymphocytes) of immune type II interferon (Epstein

et al., 1972). It is relevant that a higher titre of interferon (IF) is produced by HSV-1 resistant C57BL/6 (B6) mice than by susceptible DBA/2 (D2) mice, both in vivo and in spleen cells explanted from these mice; and further, spleen cells derived from BALB/c and A/mice were found to produce a very low level of IF compared to C57BL/6 and B6D2 F_1 hybrid mice (Kirchner et al., 1978b). Interferon can also be found at local inoculation sites such as in brain (Bostandzhyan and Bikbulatov, 1967) or in the skin (Force et al., 1965; Sydiskis and Schultz, 1966) of rabbits and mice inoculated with herpes simplex virus. Preliminary experiments revealed IF antiviral (anti VSV) activity in skin samples from HSV infected mice as early as 8 hours post infection and a high level of interferon was detected in the skin within 24 hours post infection (a time at which viral concentrations in the skin are still relatively of low levels, (Sydiskis and Schultz, 1966)).

Although during my work no attempts were made to estimate the interferon response, it can be assumed that interferon is either not produced by the susceptible mice or the level of interferon induced by HSV-2 infection is not sufficient to achieve a potent antiviral effect. It has been concluded that the early production of IF is an important element in the response of mice to several virus infections including HSV (Gresser et al., 1976). However, the role played by interferon in this context is still a matter of speculation. Recent studies (Collier et al., 1983) showed that there is no relationship between interferon production and HSV-1 replication in mouse embryo cells derived from susceptible (A-J and DBA/2) or resistant (C57BL/6) or C57BL/6 x DBA/2 (FI) mice. Higher natural killer (NK) cell activity was observed in resistant mice than in susceptible mice (Kirchner et al., 1980)

and preliminary results showed that resistant strain mice, resistant FI mice and back cross mice (resistant to challenge with 10^6 pfu of HSV-1) had spleen cells which responded significantly better than susceptible BALB/c and A-J mice; suggesting a strong correlation between NK cells and genetic resistance of mice to HSV-1 (Lopez, 1981). Mogensen (1977) has shown that resistance of mice to HSV-2-induced focal necrotic hepatitis is under genetic control and is determined by one dominant gene (or complex of closely linked genes) located on the X-chromosome. Other studies (Lopez, 1975, 1980a) indicated that resistance to HSV-1 in mice is a dominant genetic trait governed by two major loci (genes) and that other minor loci may also influence this resistance. It has also been shown that loci within the H-2 (gene complex) do not influence resistance or susceptiblity to HSV and since resistance genes do not segregate with the H-2 phenotype (in back crosses), the loci do not appear to be linked (Lopez, 1981). The data presented in this study indicate that there are considerable differences in susceptibility between mouse strains both in reaction to acute infection and also in their ability to establish a reactivable latent HSV-2 infection. As far as effect of mouse sex is concerned, both male and female mice were found to be equally susceptible to acute infection and equal in their ability to become latently infected. It should be emphasized that all the mice used were bred at the Institute of Virology and that the breeding system used was the same for all strains of mice: each breeding nucleus started with 6 pairs of mice which have been subsequently brother: sister mated for four generations after which the mice of each strain were random bred as a closed colony. It is noteworthy that paralysis or death were observed with all the other strains of

mice (Table 6) except the BIOZZI mice which are known (Biozzi et al., 1970) to be a "high antibody responder" strain (Ab/H). Their resistance to HSV-2 infection is probably determined by factors additional to their high antibody response, since specific neutralizing antibodies can be detected in mouse serum only after 6-7 days post infection with herpes simplex virus (Sydiskis and Schultz, 1966) by which time HSV infection has long been established at other sites e.g. sensory ganglia (Cook and Stevens, 1973). Further, it has been shown that humoral responses alone are of limited importance in eliminating acute infection (Openshaw et al., 1979a) and in maintaining latency (Sekizawa et al., 1980). In addition, it has been suggested that cell mediated immunity is important in clearing the primary infection whereas antibody is important in restricting the spread of virus to the central nervous system of BALB/c mice infected with HSV-1 (Kapoor et al., 1982). However, the possible involvement of antibodies, particularly in the latent stages of the infection can not be totally excluded.

Evidence that HSV-1 can persist in a latent state in sensory ganglia of man and experimental animals has been amply provided (Stevens and Cook, 1971, 1973; Bastian et al., 1972; Barringer, 1974; Warren et al., 1978; Lonsdale et al., 1979). The results in Table 7 clearly indicate that in my own experiments the HG52 strain of HSV-2 has also successfully invaded the sensory ganglia and established latent infection. It can be seen that all mouse strains were harbouring reactivable latent virus even when they were inoculated with the lower dose (10^5 pfu) of input virus. At this dose HSV-2 has been recovered with similar efficiencies from the DRGs of resistant (BIOZZI), moderately susceptible (BALB/c) and very susceptible (A)/mice. Even more striking similarities can be

observed in the recovery of HSV-2 wt virus from all strains except C57BL/6-J when the dose of the inoculum was increased to 10^6 pfu/mouse. These similarities together with the increase in number of positive mice releasing virus from their explanted DRGs confirm the dose dependency of acute and latent HSV-2 infection and indicate that mouse susceptibility to acute infection is not necessarily correlated with ability to establish latent ganglionic infection. In support of the above is that C57BL/6-J mice were resistant to 10^5 pfu of HSV-2 but moderately susceptible to 10^6 (Table 6), yet latent HSV-2 has been recovered from their DRGs at both doses (Table 7). BIOZZI mice were resistant to acute HSV-2 infection, but a high proportion (11/13 at 10^5 pfu and 6/6 at 10^6 pfu) of inoculated mice subsequently released latent viruses from their explanted DRG. These observations are in agreement with those reported by Lofgren and colleagues (1977) who found no apparent relationship between the capacity of the HSV-1 mutants to induce encephalitis and the establishment of latent infection in mice.

Here for the first time reactivation of HSV from the explanted footpad tissues of mice, three months after recovery from initial infection, has been reported. When one looks at the possible effect of mouse strain differences a similar kind of observation can be seen (Table 8) where HSV-2 has been recovered from the footpad tissues of both resistant, moderately susceptible and very susceptible mice. However, mouse strains appear to differ in their typical frequency of releasing virus from the footpad, for the susceptible (A) and moderately susceptible (BALB/c) mice were more likely to release virus from the footpad than the other strains of mice. Increasing the dose of input virus was accompanied by an

increase in the number of mice that released virus from their footpads. In addition, virus reactivation was observed to occur after a shorter time from tissues (FP and DRG) explanted from mice that had been inoculated with the higher dose (Table 7 and 8). This observation could be due to a greater number of potentially reactivable latent foci in mice that were inoculated with higher doses of input virus. Unexpectedly, however, no virus was recovered from the footpad tissue of PIRBRIGHT mice, but this could be due to the small number inoculated with 10^6 pfu compared to the relatively large number of mice inoculated with 10^5 pfu of HSV-2.

The distribution of virus releasing ganglia within animals at a given dose of infection and the proportion of animals positive appeared to begenerally related (Table 10). As can be seen from the results in Table 9 virus was never detected in the supernatant earlier than 7 days after explantation: this is good evidence that the virus subsequently detected had been truly latent rather than merely persistent. Frequently virus was reisolated after a much longer time. Histological studies (Fig. 28) showed that under the culture conditions used, a substantial proportion of neurones survive for one week but subsequently most appear to degenerate and disintegrate. It is not certain whether some latently infected neurones in the ganglia can survive longer or whether virus is also latent in another not yet recognised cell type - the fact is that the virus was first recovered in some instances as long as 4 weeks post explantation (Table 9). Warren et al (1978); Brown & colleagues (1979) also reported some very late reactivations and in addition were able by superinfection with ts mutants to rescue HSV ts+ virus from human trigeminal and vagus ganglia that had failed to release virus spontaneously after 30-45 days

in culture.

HSV-2 wt virus seems to become widely distributed in the ganglia of A, BALB/c and PIRBRIGHT mice following infection into the footpad (Table 10), a result which is consistent with the degree of susceptibility of these mice to the acute infection. The virus distribution seems to be slightly more restricted in C57BL/6-J than in BIOZZI mice. In every case, and not surprisingly, the ganglia that most frequently release virus were 5th and the adjacent 4th and 6th lumbar ganglia which innervate the footpad. Occasionally, virus was recovered from other ganglia including the sacral and thoracic ganglia. This warrants some discussion: the virus can reach ganglia other than those innervating the footpad by one of the following routes: i, virus may be spread through viremia so reaching ganglia and settling there; this route appears to be unlikely as no virus was ever recovered from the left side ganglia; moreover it would not explain why the virus is most frequently recovered from ganglia innverating the infected footpad than from other adjacent ganglia (Table 10). In addition, it has been conclusively shown that HSV travels to the CNS through the intraaxonal route (Cook and Stevens, 1973); ii, alternatively, virus may pass to other ganglia either via the spinal cord or by travelling in nerves that are in anatomical communication with the ganglia that innervate the footpad. The observed pattern of virus reactivation (Table 10) from the explanted ganglia of some mice e.g. PIRBRIGHT and A, demonstrates that the virus can be recovered from ganglia not innervating the footpad, i.e. 13th and 12th thoracic. Further, virus was also isolated from the spinal cord of two A/mice (Table 27). These observations, together with the failure to recover the virus from the spinal cord of other mice used in

the same experiment whose ganglia were releasing virus suggest that the virus may travel through the spinal cord and/or nerves. Besides, free virus was demonstrated in the spinal ganglia of mice in which either the sciatic or the femoral nerve was severed (Klein, 1982). The successful isolation of virus from the spinal cord of A/mice (2/5) and not from other strains of mice may reflect the higher susceptibility of A/mice to the infection and is in agreement with the previous observations (Nesburn et al., 1972; Knotts et al., 1973; Price et al., 1975; Cook and Stevens, 1976; Warren et al., 1978; Cabrera et al., 1980) that HSV can induce and can be recovered from the latent state both in peripheral and in central nervous tissues. No virus was recovered from the brains of mice used in this study after footpad inoculation. However, latent virus could be recovered from the sensory ganglia of mice which also released latent virus from their explanted spinal cord. No significant difference was noticed in the time of virus reactivation from sensory ganglia or spinal cord. It can not be deduced, however, whether virus recovered was directly derived from that originally inoculated in the footpad or whether the virus had replicated in nervous tissue before the establishment of the latent infection. The virus was also demonstrated in the spinal cord of PIRBRIGHT mice which had recovered from acute infection but were still paralysed 5 months post infection (Fig. 18). The result of this experiment (Table 28) indicates that latent HSV can be retrieved not only from sensory ganglia ipsilateral to the site of inoculation but also from the contralateral ganglia as well as from the spinal cord in some cases. It is not clear how the virus reached the left side ganglia. Recovery of virus from the spinal cord and from the non-inoculated side confirms the early observations

reported by Knotts and colleagues (1973) and suggests the possibility that HSV may establish latency in the central nervous system of man.

The results described here suggest that symptomatic infection is not a necessary step in the establishment of latency. No sign of illness, paralysis or death was observed in the survivors. However, it can be assumed that some viral replication and release which is restricted by the host defence system occurs first at the site of inoculation and at the level of the ganglia with subsequent prevention of the development of clinically overt disease. Evidence for the productive infection has been shown by the presence of viral mRNA (Puga et al., 1978) and by the increase of the amount of virus (Cook and Stevens, 1973) in the ganglia. However, it has also been shown that topical treatment at the site of inoculation with phosphonoacetic acid reduced the amount of virus in the ganglia (Klein and Destefano, 1981) and that (ACV^r) resistant mutants can establish acute but not latent infection in trigeminal ganglia of mice (Klein et al., 1980). Furthermore, no relationship was found between the establishment of latent infection and the development of acute encephalitis in mice inoculated with HSV-1 mutants (Lofgren et al., 1977). In support of the conclusions of McLennan and Darby (1980) that there is no absolute requirement for a productive infection during the establishment of latency in neurones is the observation that HSV-2 ts mutants were non-pathogenic when inoculated into the footpads of mice (Al-Saadi et al., 1983) and that these HSV-2 ts mutants were not able to grow at (38.5^{o}C) the non permissive temperature either in vitro (Halliburton and Timbury, 1973, 1976) or in vivo when inoculated in the mouse brain (Fig. 23). However, as will be

discussed later the mutants were able to induce latent infection in all strains of mice including the resistant BIOZZI line.

The method of detection of latent virus by sampling the supernatant which has been used in this study and by other workers (Stevens and Cook, 1971; Nesburn et al., 1972; Knotts et al., 1973; Price et al., 1975, 1976; Walz et al., 1976) has proven to be more effective than the method of homogenizing the tissue several days after explantation (Knotts et al., 1973; Price et al., 1975). Very frequently virus takes a longer time to reactivate and homogenization of explanted tissue may give an underestimated figure of reactivable viruses. The two methods, however, give no information about the type of cell in the ganglion in which the reactivation occurs, or about the possible infection of other ganglionic cells by reactivated viruses.

To get such information another approach was also used to induce reactivation of latent viruses during the course of this study. Ganglia from latently infected mice were dissected and dissociated into a single cell suspension and subsequently cultured. Latent virus was first recovered (Table 30) from the supernatant of cultured cells 3-5 days after dissociation, a much shorter period of time than that required to detect reactivated virus in the supernatants of cultured intact ganglia. The earlier detection of latent virus by the dissociation method is thought to reflect the ready access released virus has to the supernatant. It may be that in some cases virus is trapped for varying periods in intact ganglia during culture, reducing somewhat the sensitivity of this assay for latent virus reactivation. However, any such reduced sensitivity can not invalidate the differences observed in the

frequencies of reactivation of the 13 ts mutants and the wild type virus.

It was essential to pool all the ganglia from four mice in order to have a viable number of cells in culture after dissociation and consequently this approach does not allow the experimenter to determine which mouse released virus and from which ganglion. In addition, there will be some loss of cells as a consequence of the physical manipulation during dissociation and plating, which is difficult to estimate accurately. Walz and colleagues (1976), also using the dissociation and immunofluorescent technique, have reported that the number of infected ganglionic cells varies considerably in different experimental conditions. Kennedy, Al-Saadi and Clements (1983) reported the successful reactivation of HSV-1 and HSV-2 from dissociated DRG cultures of mice by the third day after dissociation of the tissue. A cytopathic effect was observed in cells of the dissociated ganglia 5-6 days post dissociation an observation which indicated that cells other than neurones were likely to be infected (at least in vitro) by reactivated virus. Moreover, immunofluorescence studies with A_2B_5 (neuronal cell marker) and anti HSV serum revealed that only a small proportion of the cell population (approximately 0.1-0.2%) were both $A_2B_5^+$ and anti HSV^+; however, a large proportion of the cell population was anti HSV^+ 4 days post dissociation, presumably due to the infection of further cells by reactivated virus. Although it is known that neurones harbour the latent virus (Cook and Stevens, 1973; McLennan and Darby, 1980), it has also been shown that both neurones and supporting cells produce virus-specific antigens during the acute phase of infection (Cook and Stevens, 1973) and that HSV could be isolated from

the trigeminal nerve roots of some human cadavers (Warren et al., 1982) possibly suggesting that the virus may also establish latency in cells other than ganglionic neurones. In addition, evidence that HSV can establish latent infection in non-neural sites of guinea pigs and mice has also been reported by Scriba (1977, 1981) and Al-Saadi et al. (1983).

In conclusion, experiments using wt of HSV-2 strain HG52 have produced the following information: 1, the virus was found to be able to establish latent infection in the sensory ganglia of the PIRBRIGHT, C57BL/6-J, BALB/c, BIOZZI and A strains of mice and in the spinal cord of both PIRBRIGHT and A strain mice; 2, the virus was also recoverable from tissues explanted from the site of inoculation (FP); 3, the establishment of latent infection and recoverability of the virus both from the DRG and FP is dose dependent; 4, the distribution of the latent virus in the ganglia of different strains of mice is correlated with susceptibility of the mice to HSV infection; 5, successful establishment of latent infection apparently is not related to the susceptibility of the mice to the acute HSV infection; 6, mouse strains differ widely in their ability to support the establishment of reactivable latent ganglionic infections; A/mice were found to be most susceptible while BIOZZI, BALB/c and PRIBRIGHT mice were relatively less susceptible and C57BL/6-J were the least susceptible; 7, virus could be reactivated from the latent state both in explanted and in dissociated sensory ganglia, HSV-specific immunofluorescence first appearing in neurones.

Temperature Sensitive Mutants

Unlike the wild type virus, HSV-2 derived temperature sensitive mutants were avirulent when inoculated subcutaneously in the footpad of the mice used in this study. None of the

inoculated mice showed evidence of illness and none died, though shortly after inoculation, swelling and reddening of the inoculated footpad was observed in some cases. The mouse core body temperature (38.5°C) is non permissive for the ts mutants, which might explain the inability of the mutants to induce symptomatic acute infection. However, the footpads may be somewhat below the core temperature when the mice are active and a possible development of microscopic lesions in the footpad has not been excluded. Nevertheless, the wild type virus (from which the ts mutants derive) never produced clinically detectable lesions in the footpad skin. Though the mutants may replicate to a limited extent at the site of inoculation the likely explanation for the lack of virulence of the mutants is the restriction in replication of the ts mutants by the non permissive temperature of the mouse core. In addition, both non specific responses such as interferon production and macrophages (particularly in the early stages of infection), as well as specific cell mediated and humoral immunity (in later stage of infection) may play a part in limiting virus spread.

In vitro studies (Fig. 24) indicated that some of the mutants grow slightly less efficiently at 36.5°C than at 34.5°C or 31°C. However, none of the mutants showed any detectable growth at the non permissive 38.5°C temperature, which is in accord with earlier reports (Halliburton and Timbury, 1973, 1976). Further, in vivo studies showed that none of the mutants inoculated were able to grow in the mouse brain (Fig. 23) so that infectious virus had become non detectable 2 hours or more after the intracerebral inoculation. Some of the mutants (ts 5 and ts 10) appeared to absorb slightly faster than the others to the mouse brain. In contrast the wt virus

which was also not demonstrable at 2 hours post infection, could be shown to be replicating subsequently for some days. Despite their inability to induce acute symptomatic infection in the mouse, when inoculated both into the brain or into the footpads, some of the ts mutants were found to be able to establish latent infection in sensory ganglia, and to be recovered from the footpad tissues as well. Thus the ts mutant virus genomes though unable to complete the lytic cycle in the DRG which are at the nonpermissive temperature, were able to travel up the nerve and enter the ganglion cell body; if the particular ts lesion does not affect a function vital for latency, then the viral genomes that travelled up the nerve fibre may have successfully established and maintained latency, even though not actually able to complete the lytic cycle. In such cases, one might expect latency to be detectable but perhaps at low efficiency; in addition this might be influenced by the dose of input virus and the number of nerve endings exposed to the virus at the site of inoculation. As was the case after infection with wt virus, mouse strains varied in their ability to become latently infected by the ts mutants, and between different ts mutants differences in establishing reactivable latency were also observed. It can be concluded from the cumulative mutant totals (see Discussion Table 1 at the end of this chapter) that the strains most prone to reactivable latent infection in the ganglia were BALB/c and A/mice; least prone were mice of the PIRBRIGHT strain while C57BL/6-J and BIOZZI mice were intermediate. On the whole these results are in reasonable agreement (bearing in mind the small number used) with results obtained with the wild type virus. The following conclusions can be drawn from the analysis of the results presented in Discussion Table 1:

(a) the mutants behaved differently. They are heterogeneous

(b) they can be subdivided into at least three subgroups; the first group of low recoverability comprised of ts 3, 5 and 9, the second group (high recoverability) consists of ts 1, 4 and 8, while the rest of the mutants are members of the middle group, however, it is not clear whether they constitute one group (middle) or indeed some may belong to the lower or higher group. Larger numbers of samples would be required to ascertain the state of the mutants of the middle group.

(c) if one takes the wild type virus into consideration it would fall into another subgroup

(d) there is evidence that some mutants behave sometimes differently in different mouse strains for example ts 7, 8, 4, 2 and 12; or similarly ts 3 and 5; ts 1 and 8. This is ts mutant: mouse strain interaction

(e) even in the two highly prone strains of mice A and BALB/c, some mutants behave very differently e.g. ts 2, 7. This is also true for C57BL/6-J and BIOZZI e.g. ts 2 and 4

With respect to mouse strain exactly similar conclusions can be drawn from the data concerning the recovery of the ts mutants from the footpads (see Discussion Table 2). The order from most to least frequent was BALB/c> A> C57BL/6-J, BIOZZI> PIRBRIGHT. Statistical analysis, using the multiple comparison procedures of Cox (1970) which has an overall significance level of 0.05, confirmed the following conclusions:

(a) the mutants can be subdivided into three subgroups: the first group consists of two members i.e. ts 5

and ts 11 which were not recovered from the footpad; the second group has one member, ts 3, that has been frequently recovered from the footpad. The third group is the middle group comprised of the rest of the ts mutants. As in the case of their recovery from the DRG, larger samples of these middle group members would be required to ascertain their state.

(b) unlike its recovery from the DRG the wild type virus falls within the middle group of viruses recovered from the footpad

(c) some mutants behave similarly, for example ts 5 and ts 11, have never been recovered from the footpad of any of the mouse strains, or ts 2 and ts 3 which were recovered even from the footpad of the least prone strain of PIRBRIGHT mice

(d) also there is evidence that some mutants behave differently in different mouse strains, for example ts 10 and ts 11 in BALB/c and A mice but not in PIRBRIGHT, C57BL/6-J and BIOZZI; or the behaviour of ts 1 in the two highly prone strains of mice A and BALB/c. Again this is ts mutant: host interaction

Thus reactivation from latency is least frequently obtained in the PIRBRIGHT strain of mice both from explanted DRG and FP, most frequently from BALB/c and A strains and intermediate for BIOZZI and C57BL/6-J. However, with the number of mice used in this study, it was not possible to demonstrate a statistically significant difference between BALB/c, A, BIOZZI & C57BL/6-J mice strain at the DRG level or between PIRBRIGHT, BIOZZI & C57BL/6-J at the FP level. In general it can be concluded that the pattern of the infection seems reproducible in each particular strain of mice and that

host factors influence the incidence of reactivable latent infection at either site (FP and DRG). As will be shown later, reactivable latent infection can be established at both sites (FP and DRG) independently.

In addition, to these mouse strain-dependent differences in response to ts mutant infection, striking variations distinguishing the various ts mutants were also observed (Discussion Table 1). Each of the mutants was found to behave in a characteristic way (in respect of ability to establish a reactivable latent infection in the sensory ganglia) in a particular mouse strain: for example the results of ts 1, ts 8, ts 10 and ts 11 are in total agreement with the overall classification of mouse strains into highly susceptible (BALB/c and A) least susceptible (PIRBRIGHT) and intermediate (C57BL/6-J and BIOZZI) whereas the results observed with ts 2 and ts 7 would lead to quite different hierarchies; A >BIOZZI >BALB/c, C57BL/6-J, PIRBRIGHT and PIRBRIGHT, BALB/c >BIOZZI >C57BL/6-J, A. Mutants ts 3 and ts 5 were only recovered from the sensory ganglia of a single C57BL/6-J mouse each (even though ts 3 had been inoculated into as many as 24 A/strain mice). Mutant ts 9 was only recovered once but in this case from an A/mouse. Clearly these three mutants are each defective in different functions that affect latency. Mutants ts 6, 10, 11 and 13 were also only infrequently recovered and seem to modify the potential of the virus to induce latency.

Ts 4, 12 and 13 differ somewhat in their reactivation patterns: ts 4 was found to induce reactivable latent infection more efficiently in the sensory ganglia of BIOZZI mice than in the other mouse strains, while ts 12 was readily recovered from all other strains except BIOZZI. Ts 13 was not recovered from the sensory ganglia of C57BL/6-J or BALB/c mice, only once from

the DRG of A/mice and twice each from PIRBRIGHT and BIOZZI. It is interesting that the ts mutants also differ in their distribution within the ganglia of particular mouse strains (see Discusion Table 4); those mutants that were recovered from all mouse strains (ts 1, ts 4, ts 6 and ts 8) were found in a wider range of ganglia in the mouse strains where they induced a high level of latent infection and were more restricted in distribution within the ganglia of mice from strains where the level of latent infection was low. It can be seen that in the most prone BALB/c and A strain mice the mutants were occasionally being recovered from the 3rd and often from the 4th lumbar ganglia, whereas no virus was ever recovered from a ganglion beyond the 4th lumbar in other less prone strains (C57BL/6-J and BIOZZI) of mice. The mutants were even more restricted to the 6th and 5th lumbar ganglia in PIRBRIGHT mice. In all strains of mice the mutant viruses as well as the wild type were most frequently recovered from the 5th and 6th lumbar ganglia, which indicates that these two ganglia were the main target sites for the establishment of reactivable latent infection following the footpad inoculation. The combined experimental observations indicate conclusively, that the sequelae of virus infection are influenced by the host genetic makeup and equally importantly the virus genotype.

Analysis of the data (Discussion Table 1) revealed that the ts mutants also vary in their overall ability to establish latent infection in the sensory ganglia of mice. Some mutants, viz, ts 1, ts 4 and ts 8 were found to be of high latency capacity and as a result virus was recovered from ganglia of >37% of the inoculated mice. These mutants were able to induce latent infection in all five strains of mice; one other mutant, ts 6, was also able to establish latent infection in all

strains of mice though at a much lower level (~10%). This mutant is known to have a temperature sensitive DNA polymerase (Hay et al., 1976). The latency capacity of ts 2 and ts 12 was fairly high so that about one fourth of the inoculated mice could be shown to harbour latent virus. Ts 7 was fairly easily reactivated from PIRBRIGHT, BALB/c and BIOZZI mice, but not at all from A or C57BL/6-J. Ts 6, ts 10, ts 11 and ts 13 were each recovered from about 10% of the mice. The remaining mutants, ts 3, ts 5, ts 9 were severely limited in the ability to establish latent infection in the DRG and clearly are each genetically impaired in functions which affect latency as well as ability to replicate at non permissive temperature. In general, the mutants were much less efficient than the wt in the establishment of the latent infection in the DRG. However, in some mouse strains some mutants were found to possess a similar capacity to that of the wt virus; for example ts 4 in the BIOZZI and ts 8 in the BALB/c mice suggesting that certain levels of latency can be experimentally controlled by selecting the appropriate host and virus. It is important to note that the mutants were inoculated at 10^5-10^6 pfu/mouse, however, the variations in the latency capacity of the mutants seems not to be determined simply by the dose of the input virus; since viruses inoculated at the lower input dose (10^5 pfu) were found to establish the latent infection with high efficiency (ts 1 and ts 8); similarly some mutants inoculated at high input dose (10^6 pfu/mouse) were found not efficient (ts 5) or of poor efficiency (ts 6) in the establishment of reactivable latent infection. All mutants used in this study survived reasonably when incubated at 31°C (Fig. 25) and thus loss of virus by thermal inactivation after shedding and before sampling is not thought to have contributed materially to the results. It can

be argued that only a very small amount of virus may be released during each incident of reactivation, but that released viruses are then amplified by replication in the supporting cells within the ganglion culture.

Recovered viruses must have passed through the classical 3 stages of successful latent infection, i.e. initiation of latency, maintenance and reactivation. Besides, the viruses must have travelled from the site of inoculation (FP) to the sensory ganglia. The fact that ts 3, ts 5 and ts 9 were reactivated in the sensory ganglia, though only on one occasion each, precludes the assumption that these mutants were not able to reach the ganglia or were totally incapable of the reactivation process. In addition, ts 3 and ts 9 have been reactivated and recovered quite readily from the footpad tissue 3 months after inoculation. Hence, it seems that these mutants are deficient in functions essentially involved in the establishment and/or maintenance of the latent infection in the sensory ganglia. Additional evidence for the deficient capacity for latency of ts 3 is the observation that no virus was recovered and no immunofluorescence positive foci were observed in cultures of dissociated explanted ganglia from mice inoculated with the virus (Table 30). It was important to determine the latency capacity of these particular mutants by inoculating them directly into the tissues of interest (i.e. nervous tissue) and since the DRG were not accessible, the mouse brain was chosen for this purpose. The particle/infectivity ratio was determined for ts 3, ts 5 and ts 9 and found to be 48, 54 and 102 respectively, and the growth of these mutants compared with that of the wt virus in the mouse brain was studied. None of these mutants were able to grow. Further, brains from inoculated mice (six weeks after

the intracerebral inoculation) were removed and cultured as described. The results (Table 29) showed that none of the mutants (ts 3, ts 5 and ts 9) were recoverable despite the increase in the dose inoculated (ts 5 and ts 9) from 10^5 to 10^6 pfu/mose brain. On the other hand, ts 6 which is of low latency capacity in the DRG, was recovered from the latent state in the mouse brain.

HSV-2 wt was recovered from the footpads (Discussion Table 2) with approximately comparable frequency as some of the ts mutants, although some mutants (ts 3 and ts 10) appeared possibly superior to wild type. On the other hand ts 5 and ts 11 were not recovered from any culture of the explanted footpad tissues. Recovery of other mutants ranged from 6-9% for ts 6, ts 7 and ts 12 and from 11-16% for ts 1, ts 2, ts 8, ts 9 and ts 12 (Discussion Table 2). As was the case with their recovery from the sensory ganglia, differences were observed in the frequencies of recovery of the mutants (FP) between strains of mice; for example ts 2 and ts 3 were recovered from the footpad tissues of all strains of mice, whereas other mutants either were not recovered at all (ts 5 and ts 11) or recovered from some, but not all strains of mice. Further, the frequency of recovery of each mutant appeared to be different in each particular mouse strain e.g. ts 1 in BALB/c and BIOZZI mice or ts 4 in the first four strains of mice compared to A/mice. The differences between the viruses were statistically significant and analysis of the data leads to the conclusion that ts 3 is more prone than most other used mutants (particularly ts 5 and ts 11) to be recovered from the explanted FP.

It is relevant to discuss the subject of virus recovery from the two sites i.e. the dorsal root ganglia and the footpad

together. Interestingly, some mutants (ts 3, ts 9 and ts 10) have been recovered more frequently from the footpad than from the ganglia, however the majority of the viruses including the wt virus were recovered more often from the ganglia. Of special significance is the observation that ts 5 and ts 11 were never recovered (0/59 and 0/58) from the footpads and only once recovered (ts 5) or recovered to a limited extent (ts 11) from the dorsal root ganglia. This suggests that the impairment of ts 5 affects almost completely its recovery from both DRG and FP whereas that of ts 11 has its main effect on the recovery from FP.

Further analysis of the data, where the recovery of the mutants from the DRG and FP in individual mice was compared, revealed that viruses were either recoverable both from the DRG and FP, or only from the DRG, or only from the FP (Discussion Table 3). Remarkably viruses (including wt virus) were only in a minority of cases recovered from both the footpad and DRG of the same mouse. The viruses were much more often independently recovered either from the ganglia or from the footpads; for example, ts 11 was recovered from the sensory ganglia and never from the footpads while ts 3 and ts 9 were recovered from the footpad tissues very much more frequently than they were recovered from ganglia. In fact, ts 3 was recovered from the ganglia of only 1/16 mice but from 15/16 of the footpads of these mice (Discussion Table 3). Similarly, ts 9 and ts 10 were both recovered from the footpads of 7/8 and 9/15 mice respectively compared to 1/8 and 4/15 from the ganglia. Moreover, none of the mutants, ts 3, ts 6 and ts 9 were ever recovered from the DRG and FP of the same mouse. The number of mice which released virus from both their DRG and FP was overall 24/195. It is noteworthy that the number of mice

releasing virus from their DRG only was 108/195 and from the FP was 63/195 (see Discussion Table 3).

The recovery of wt as well as ts mutant viruses from footpad tissue both from resistant and susceptible mouse strains suggests that the host defense responses do not correlate with recoverability from this site. Recovery of virus from the peripheral regions may be due either to reactivation of virus latent within nervous tissues (DRG) which subsequently travels via a nerve to the peripheral site, or to the presence of virus, over a long period of time either persistent (slowly replicating) or latent at the peripheral site. However, no virus was ever released from the FP earlier than 8 days after explantation and no virus was ever recovered immediately after explantation from homogenised FP (Table 31). No symptoms of reactivation in the footpad were ever detected. Moreover, it has been concluded (McLennan and Darby, 1980) that virus reactivation will eventually lead to the death of the infected cell, presumably due to virus replication. The mutants were not able to grow at the non permissive (38.5^{o}C) temperature (this thesis and Halliburton and Timbury, 1973, 1976); hence viruses recovered from the footpad could not have undergone a normal replication process involving the complete lytic cycle in vivo within the mouse sensory ganglia. It is however possible to imagine that the genomes of some of the mutants could have passed down the nerve fibre to the periphery without becoming enveloped into an infectious virus particle, but there is no evidence for this. The evidence therefore strongly favours some form of latency at the peripheral site. As neurone cell bodies have never been described as located in the mouse footpad, cells other than neurones must shelter the latent virus. In accord with the latter conclusion is the

difference in time of virus reactivation from the ganglia and from the footpads. Both the wild type and ts mutant viruses required a longer time in order to reactivate from the footpad than from the ganglia (Discussion Table 5) and in some cases (for example the wt and ts 7 in BIOZZI mice, ts 2 and ts 3 in PIRBRIGHT and C57BL/6-J mice, ts 10 in A/mice or ts 1 in BALB/c mice) viruses were not recovered from the footpad cultures until 32-45 days post explantation. In contrast, the latest time of virus reactivation from the ganglia in these experiments was 28 days post explantation. Of course, the possibility that mechanical trapping after reactivation from the footpad tissue but before release of virus into the medium might contribute to the differences in timing, cannot be excluded. It does not seem very likely that trapping alone could lead to the large and consistent difference observed.

It would be important to determine the type of cells that were (possibly) latently infected at the footpad, and it would be interesting to compare the mechanism of latency in both types of cell (i.e. the ganglion sensory neurones and the non neuronal cells in the FP). The use of in situ hybridization and immunofluorescent techniques should identify the types of cells involved in the lytic cycle at the FP and eventually may also determine the latently infected cells though positive immunofluorescence is not necessarily to be expected during the true latent phase of infection. Such experiments remain to be done.

Reactivated viruses both from the DRG and FP were randomly chosen and their DNA restriction enzyme profiles were compared to that of the standard HSV-2 and to the ts mutant viruses initially used for inoculation.

Comparative studies of restriction endonuclease (RE)

digests of the DNA of different strains of HSV-1 and HSV-2 revealed that the two subtypes can be readily differentiated (Lonsdale, 1979) and that strains exhibited characteristic profile within each subtype (Hayward et al., 1975a; Skare et al., 1975). Variation in RE patterns within a subtype could result from: 1) loss and/or gain of restriction sites, due to point mutation within the restriction site or due to small deletions or insertions; 2) size (mobility) variability of restriction fragments probably due to a variable copy number of tandemly reiterated sequences within these fragments. Both types of variations have been observed in viruses recovered from human ganglia (Lonsdale et al., 1979, 1980; Chaney et al., 1983). However, studies of clonally related isolates very rarely showed instances of the loss or gain of restriction endonuclease cleavage sites (Lonsdale et al., 1980). On the other hand, size variability of particular restriction fragments was regularly observed both in isolates prepared by plaque purification of parent strain and in isolates from different clinical episodes, including reactivated viruses.

In the present study, changes in RE cleavage pattern were most frequently confined to the size variability of restriction fragments, particularly Bam HI g, p, u, w, z, a'. The fragments Bam p, z and a' are known to contain tandemly reiterated DNA sequences (Lindsay Whitton - personal communication) and their variation in mobility is most probably due to variation in copy number of the tandem repeats, a phenomenon previously observed in HSV-1 (Davison et al., 1981) and in human genomic DNA (Spritz, 1981). It is not known whether the other variable fragments contain similar tandem reiterated sequences.

In one case, the loss of a restriction site (Hind III

m-1) was observed (Figure 26-d). This is the first demonstration that this phenomenon which has previously been shown to occur in HSV-2 strains freshly isolated from the human population (Chaney et al., 1983) can be experimentally reproduced by a single-latent-passage of HSV-2 in mice. This single observation among the studied isolates supports the proposal that loss or gain of a restriction site is a relatively rare event during virus growth (Lonsdale et al., 1980; Roizman and Tognon, 1982). It is unknown at which stage of the whole latency producing process these alterations in the viral genome occurred. No significance could be attached to the differences between RE profiles of virus isolated from the footpad and from the ganglia of individual mice.

The viral genome appears to be latent within the sensory neurones of the ganglia (McLennan and Darby, 1980; Cook and Stevens, 1973) and certain stimuli applied to the area of the latent infection, such as epinephrine iontophoresis (Kown et al., 1981; Hill et al., 1982) or surgical manipulation (Walz et al., 1974; McLennan and Darby, 1980; Klein, 1982) have been shown to lead to the reactivation of virus with subsequent shedding at the periphery. Virus, however, has also been recovered from central nervous tissue, autonomic ganglia and the adrenal gland (Nesburn et al., 1972; Knotts et al., 1973; Price et al., 1975; Cook and Stevens, 1976; Warren et al., 1978). Skin explants from the sites, in man, at which HSV recurrences have been observed have never yielded virus, however these attempts have not been repeated recently and it is noteworthy that only the superficial layers of the skin were cultured (Rustigan et al., 1966). However, recurrent herpetic lesions have been observed in patients with 'blow out' fractures which had severed the nerve supply to the area of

skin involved (Hoyt and Billson, 1976).

Further, HSV has been shown to be present in peripheral tissues of latently infected animals. Scriba (1976, 1977) isolated HSV-1 and HSV-2 from the site of inoculation (footpad and vagina) of latently infected guineag pigs; Donnenberg and colleagues (1980) have also isolated HSV-2 from guinea pig footpad. HSV-1 has been isolated from the site of inoculation (i.e. ear skin) in 8% of latently infected mice in the absence of overt clinical lesions (Hill et al., 1980). Recently, Park and Macnab (1983) have suggested that in the inbred Hooded Lister rat, the fibroblast cells (transformed or tumour cells) has the capacity to harbour non replicating HSV genomes. It is not precisely known what viral gene(s) functions are essential for the successful establishment of the latent infection, however, studies with HSV-1 mutants (Watson et al., 1980) have already clearly indicated that at least one immediate early and one or more later virus genes are necessary for the establishment and/or maintenance of the latent state. Comparison of latency characteristics with the phenotypic properties of the mutants expressed under the restrictive conditions (Halliburton and Timbury, 1973; Macnab, 1974; Halliburton and Timbury, 1976; Hay et al., 1976; Moss et al., 1979; Moss, 1982) revealed that there is no correlation between the latency phenotype and viral DNA synthesis, DNA polymerase and DNAase activity, or cell transformation ability. For example, ts 3 is known to synthesize a limited amount of viral specific polypeptides at the non permissive temperature, while ts 5 synthesized normal amounts of the viral polypeptides, both mutants are DNA^+ and both were found to be severely limited in the ability to establish a reactivable latent infection in the mouse DRG. On the other hand, ts 9 is a DNA^- virus and have the

earliest block (see Table 1) synthesizing very little viral polypeptides in BHK cells at the non permissive temperature. Ts 9 was also found to be defective in the establishment of reactivable latent infection in the sensory ganglia. Of the three mutants, ts 3 was recovered very frequently from the footpad tissues while ts 5 was never recovered from the footpad. It is noteworthy that the above virus-specific activities were measured in virus-infected BHK cells, and it is not known how relevant is the behaviour of these mutants in BHK cells to their behaviour in the differentiated cells of the hosts. However, these observations confirm and extend the conclusions reported by Watson and colleagues (1980) and Stevens (1981) that viral genes essential for the establishment of latent infection are not confined or clustered on the physical and genetic map of HSV. For the time being, there is not sufficient information to allow possible correlation of the latency characteristics of the viruses to their phenotypes and it is extremely important to better characterise the ts mutations of these HSV-2 ts mutants. It has been reported (Tenser and Dunstan, 1979) that TK gene expression is important in the pathogenesis of HSV infection and necessary for the establishment of the infection in the sensory ganglia, however, some TK^- mutants were able to establish latent infection, but with more difficulty, in the ganglia of mice (Field and Wildy, 1978; Tenser and Dunstan, 1979; Field and Darby, 1980). The results presented in this study showed that some thymidine kinase positive (TK^+) mutants (namely ts 3, 5 and 9) were also very deficient in the establishment of the latent infection in sensory ganglia. This indicates that in the presence of intact TK&other gene functions are also important and perhaps essential for the viral genome to pass through all the stages

involved in a latent infection.

The ts mutants were found to grow, in vitro, at 36.5°C (2 degrees below the non permissive temperature) and it is not possible to exclude the possibility that some leakiness allowing replication may have occurred at times in the mouse footpad; any leak-through, however, would tend to increase the capacity for viral gene expression and therefore favour the establishment of latency. These considerations cannot help to explain the non recovery of mutants ts 5 and ts 11 from the footpad or the recovery of ts 3, ts 5 and ts 9 (only on one occasion each) from the sensory ganglia.

The results of this study using HSV-2 (HG52) ts mutants leads to the following conclusions:-

1. non recovery of ts 5 from the footpad and its single recovery (1/59) from the sensory ganglia suggest that this mutant is deficient in functions either necessary for establishment or maintenance of latency in mice or for reactivation
2. recovery of some mutants (ts 3 and ts 9) from the footpad at frequencies (15/56, 7/53) much greater than their recovery from the sensory ganglia (1/80, 1/66) and the recovery of ts 5 and ts 11 only from the DRG support the notion of independent establishment of latency in the footpad and DRG and imply that these viruses can go latent in tissues other than the ganglia or the CNS
3. only a minority of mice produced virus both from the footpad and ganglia. This is in keeping with the hypothesis that HSV-2 can induce latent infection independently at the two sites
4. the observed differences in successful reactivation between HSV-2 ts mutants from the DRG and from the FP, are also

evidence that the genetic control of the ability of HSV to produce latent infection is influenced by several viral genes. Observations of ts mutants in different genes with impaired latency give us a minimum estimate of genes essential (ts 5) and genes that modify latency

5. differences in susceptibility of mouse strains to the latent infection underline the important roles of host (genetically determined) factors in modulating the events involving virus - host interaction leading to the establishment of latency and recovery of virus
6. the RE study also suggests that modifications of the viral DNA can occur during one or more of the stages in the process of initiating, maintaining or reactivating from latency.

As a result of these studies, the mouse model of HSV-2 latency has to be modified so as to include the footpad tissues as another possible site for latent HSV infection in addition to the DRG.

It has been suggested that stimuli to the sensory neurone will result in reactivation of the latent virus from the ganglion cells presumably due to the direct effect of the stimulus on the neurone (ganglion trigger theory) or indirectly by acting on the skin area (skin trigger theory) innervated by that neurone (Hill and Blyth, 1976). It is now necessary to postulate, the presence of virus latent at the periphery in a cell other than the neurone, and that stimuli causing virus reactivation at this site may either act directly on the skin or via the sensory neurones or a combination of the two. Virus can therefore reactivate independently from either DRG or the footpad.

In humans, herpes simplex virus has never been recovered

from the skin or other peripheral tissues of apparently healthy individuals. However, it should be noted that in the experiments described in this thesis the whole layers comprising the skin tissue were excised from the footpad and that HSV took a long time to reactivate from the footpad. In addition, the total number of footpads checked during this study was not less than 725 for the ts mutants and 81 for the wild type (Discussion Table 2). Thus previous negative results in man (Stadler and Zurukzoglu, 1936; Findlay and MaCullum, 1940; Rustigan et al., 1966) may have a trivial explanation and could be of questionable significance. HSV recurrences on the face occur at sites where biopsy would be difficult to justify ethically as a reasonable experimental procedure that could treat the patient. Of course, it would be of value to examine the skin from other sites between recurrences in humans - for example the genital region. The possibility of latent virus in peripheral tissue of man is given credence by some clinical reports of recurrences repeatedly at exactly the same anatomical site with the same prodromal symptoms.

In experimental animals, however, the virus has been recovered from guinea pig footpad and vagina (Scriba, 1976, 1977) and from the mouse footpad (Al-Saadi et al., 1983 and the work in this thesis). In addition, HSV genomes have been recovered (by superinfection) from in vitro HSV transformed cells that had been inoculated into rats with subsequent tumour production (Park and Macnab, 1983). Analysis of the recovery of HSV genomic material from such sites may shed some light on the relationship between HSV-2 and cervical cancer in the human and could help us to gain a better understanding of long term interactions between viral sequences and host cells.

The observation that HSV-2 can be reactivated from the

footpad tissue of the mouse does open a new area for further research and could be of clinical importance, and ultimately of help in our understanding of herpes simplex virus as a disease causing agent.

DISCUSSION TABLE 1

RECOVERY OF VIRUSES FROM DRG OF DIFFERENT MOUSE STRAINS

Virus & pfu/mouse		DRG +VE/TOTAL											
		PIRBRIGHT		C57BL/6-J		BIOZZI		BALB/c		A		Cumulative data	%
ts1	$1x10^5$	1/17	(5.8%)	5/12	(41.6%)	4/11	(36.3%)	8/13	(61.5%)	7/13	(53.8%)	25/66	37.8
ts2	$1.7x10^6$	0/14	(0%)	0/14	(0%)	8/17	(47%)	1/13	(7.6%)	8/9	(88.8%)	17/67	25.3
ts3	$1.2x10^5$	0/14	(0%)	1/12	(8.3%)	0/16	(0%)	0/14	(0%)	0/24	(0%)	1/80	1.2
ts4	$5x10^6$	2/15	(13.3%)	4/13	(30.7%)	12/14	(85.7%)	11/27	(40.7%)	6/15	(40%)	35/84	41.6
ts5	$1.7x10^6$	0/13	(0%)	1/12	(8.3%)	0/12	(0%)	0/11	(0%)	0/11	(0%)	1/59	1.6
ts6	$1x10^6$	1/15	(6.6%)	2/12	(16.6%)	1/16	(6.2%)	1/15	(6.6%)	2/8	(25%)	7/66	10.6
ts7	$8x10^5$	6/15	(40%)	0/12	(0%)	2/14	(14.2%)	5/15	(33.3%)	0/13	(0%)	13/69	18.8
ts8	$2.5x10^5$	1/14	(7.1%)	4/13	(30.7%)	5/14	(35.7%)	8/9	(88.8%)	7/8	(87.5%)	25/58	43.1
ts9	$7.5x10^5$	0/19	(0%)	0/13	(0%)	0/11	(0%)	0/11	(0%)	1/12	(8.3%)	1/66	1.5
ts10	$2.5x10^5$	0/15	(0%)	0/12	(0%)	0/13	(0%)	4/12	(33.3%)	2/10	(20%)	6/62	9.6
ts11	$4.2x10^5$	0/12	(0%)	1/13	(7.6%)	1/12	(8.3%)	4/11	(36.3%)	2/13	(15.3%)	8/61	13.1
ts12	$5x10^5$	3/14	(21.4%)	5/11	(45.4%)	0/14	(0%)	5/10	(50%)	4/13	(30.7%)	17/62	27.4
ts13	$6x10^5$	2/11	(18.1%)	0/7	(0%)	2/14	(14.2%)	0/10	(0%)	1/11	(9%)	5/53	9.4
Total (mutants only)		16/188	(8.5%)	23/156	(14.7%)	35/178	(19.6%)	47/171	(27.4%)	40/160	(25%)	161/853	18.8
wt	$1x10^5$	9/15	(60%)	4/10	(40%)	11/13	(84.6%)	2/3	(66.6%)	4/4	(100%)	30/45	66.6
	$1x10^6$	6/6	(100%)	8/13	(61.5%)	6/6	(100%)	6/6	(100%)	5/5	(100%)	31/36	86.1
Total (wt only)		15/21	(71.4%)	12/23	(52.1%)	17/19	(89.4%)	8/9	(88.8%)	9/9	(100%)	61/81	76.5

Statistical analysis of the data of the recovery of TS mutants from the ganglia+

(A) Comparison of mouse strains

Group	Group	Int. Est.	
1	2	-1.5603	0.3405
1	3	-1.8382	-0.0622*
1	4	-2.2448	-0.5222*
1	5	-2.1355	-0.3779*
2	3	-1.1565	0.4759
2	4	-1.5606	0.0135
2	5	-1.4529	0.1594
3	4	-1.1437	0.2772
3	5	-1.0380	0.4251
4	5	-0.5721	0.8257

Estimated Probabilities

0.0851 0.1474 0.1966 0.2749 0.2500

Where group 1 = PIRBRIGHT
2 = C57BL/6-J
3 = BIOZZI
4 = BALB/c
5 = A/MICE

0.09 0.15 0.20 0.25 0.27

1 2 3 5 4

Conclusion

Group 1 (PIRBRIGHT) is significantly smaller in terms of proportion of positive than group 3, 5 and 4.

+ The method used to compare the strains of mice and the ts mutant viruses, involved a Bonferroni multiple comparisons procedure using empirical logistic transformation of COX (1970) on the number of 'positive' for each strain and virus. The procedure has an overall significance level of 0.05.

* signficant difference

continued/....

(B) Comparison of ts mutants

Group	Group	Int. Est.	
1	2	-0.7195	1.8651
1	3	0.8434	6.1231*
1	4	-1.3102	1.0011
1	5	0.5213	5.8318*
1	6	0.0097	3.1584*
1	7	-0.4110	2.3000
1	8	-1.4725	1.0442
1	9	0.6405	5.9386*
1	10	0.0298	3.3210*
1	11	-0.1752	2.8804
1	12	-0.8342	1.7712
1	13	-0.0534	3.4330
2	3	0.2412	5.5799*
2	4	-1.9488	0.4942
2	5	-0.0808	5.2883
2	6	-0.6119	2.6345
2	7	-1.0403	1.7838
2	8	-2.1059	0.5321
2	9	0.0384	5.3952*
2	10	-0.5898	2.7951
2	11	-0.7988	2.3580
2	12	-1.4657	1.2572
2	13	-0.6704	2.9045
3	4	-6.2438	-1.0319*
3	5	-3.8431	3.2296
3	6	-4.7159	0.9175
3	7	-5.2392	0.1618
3	8	-6.3505	-1.0444*
3	9	-3.7254	3.3380
3	10	-4.6650	1.0493
3	11	-3.9216	0.6603
3	12	-5.6892	-0.3404*
3	13	-4.7079	1.1210
4	5	0.7096	5.9526*
4	6	0.2219	3.2554*
4	7	-0.1891	2.3873
4	8	-1.2450	1.1259
4	9	0.8289	6.0594*
4	10	0.2394	3.4206*
4	11	0.0388	2.9795*
4	12	-0.6094	1.8556
4	13	0.1530	3.5357*
5	6	-4.4236	1.2386
5	7	-4.9476	0.4835
5	8	-6.0591	-0.7223*
5	9	-3.4302	3.6562
5	10	-4.3725	1.3703
5	11	-4.6295	0.9816
5	12	-5.3976	-0.0185*
5	13	-4.4151	1.4416
6	7	-2.3135	1.0345
6	8	-3.3945	-0.2019*
6	9	-1.1198	4.5308
6	10	-1.8251	2.0078
6	11	-2.0478	1.5849
6	12	-2.7471	0.5160
6	13	-1.8951	2.1066

7	8	-2.5397	0.2223
7	9	-0.3644	5.0545
7	10	-1.0103	2.4721
7	11	-1.2222	2.0384
7	12	-1.8576	0.9433
7	13	-1.0884	2.5790
8	9	0.8415	6.1659*
8	10	0.2229	3.5562*
8	11	0.0163	3.1172*
8	12	-0.6466	2.0119
8	13	0.1409	3.6670*
9	10	-4.4798	1.2515
9	11	-4.7366	0.8627
9	12	-5.5049	-0.1376*
9	13	-4.5225	1.3230
10	11	-2.2013	1.5556
10	12	-2.9074	0.4935
10	13	-2.0431	2.0718
11	12	-2.4708	0.7026
11	13	-1.6273	2.3017
12	13	-0.5737	3.0163

continued/...

Estimated probabilities

0.3788	0.2537	0.0125	0.4167	0.0169	0.1061
0.1884	0.4310	0.0152	0.0968	0.1311	0.2742
0.0943					

Where group 1-13 represents the mutants viruses (ts1-ts13).

0.01	0.02	0.02	0.09	0.10	0.11	0.13	0.19	0.25	0.27	0.38	0.42	0.43
ts 3	ts 9	ts 5	ts 13	ts 10	ts 6	ts 11	ts 7	ts 2	ts 12	ts 1	ts 4	ts 8

Conclusions

1. ts 3 and ts 9 have significantly lower proportion of positive than ts 1, 2, 4, 8 and 12

2. similarly ts 5 is significantly lower in terms of proportion of positive than ts 1, 4, 8 and 12

3. ts 4 and ts 8 are also different from ts 6, 10, 11 and 13, however larger number of samples require to ascertain the exact state of the middle group of the mutants (see Discussion)

* significant difference

N.B. the estimated probability for the wild type = 0.75

DISCUSSION TABLE 2

RECOVERY OF VIRUSES FROM THE FOOTPAD OF DIFFERENT MOUSE STRAINS

Virus & pfu/mouse		FP+VE/TOTAL											
		PIRBRIGHT		C57BL/6-J		BIOZZI		BALB/c		A		Cumulative data	%
ts1	$1x10^5$	0/11	(0%)	0/11	(0%)	2/10	(20%)	5/12	(41.6%)	0/11	(0%)	7/55	12.7
ts2	$1.7x10^6$	1/11	(9%)	2/8	(25%)	1/16	(6.2%)	2/6	(33.3%)	2/9	(22.2%)	8/50	16
ts3	$1.2x10^5$	1/6	(16.6%)	1/9	(11.1%)	5/15	(33.3%)	3/11	(27.2%)	5/15	(33.3%)	15/56	26.7
ts4	$5x10^6$	0/15	(0%)	1/13	(7.6%)	1/14	(7.1%)	3/9	(33.3%)	8/14	(57.1%)	13/65	20
ts5	$1.7x10^6$	0/13	(0%)	0/12	(0%)	0/12	(0%)	0/11	(0%)	0/11	(0%)	0/59	0
ts6	$1x10^6$	0/14	(0%)	1/12	(8.3%)	1/16	(6.2%)	1/15	(6.6%)	3/13	(23%)	6/70	8.5
ts7	$8x10^5$	0/17	(0%)	1/10	(10%)	1/11	(9%)	2/10	(20%)	0/11	(0%)	4/59	6.7
ts8	$2.5x10^5$	0/12	(0%)	1/6	(16.6%)	1/9	(11.1%)	4/9	(44.4%)	1/8	(12.5%)	7/44	15.9
ts9	$7.5x10^5$	0/12	(0%)	3/13	(23%)	2/11	(18.1%)	2/9	(22.2%)	0/8	(0%)	7/53	13.2
ts10	$2.5x10^5$	0/11	(0%)	0/12	(0%)	0/9	(0%)	6/12	(50%)	5/10	(50%)	11/54	20.3
ts11	$4.2x10^5$	0/12	(0%)	0/12	(0%)	0/12	(0%)	0/10	(0%)	0/12	(0%)	0/58	0
ts12	$5x10^5$	1/12	(8.3%)	3/10	(30%)	1/14	(7.1%)	0/4	(0%)	1/11	(9%)	6/51	11.7
ts13	$6x10^5$	1/9	(11.1%)	0/7	(0%)	0/14	(0%)	1/10	(10%)	2/11	(18.1%)	4/51	7.8
Total (mutants only)		4/155	(2.5%)	13/135	(9.6%)	15/163	(9.2%)	29/128	(22.6%)	27/144	(18.7%)	88/725	12.1
wt	$1x10^5$	3/22	(13.6%)	1/4	(25%)	1/14	(7.1%)	1/5	(20%)	NA		6/45	13.3
	$1x10^6$	0/6	(0%)	4/13	(30.7%)	2/6	(33.3%)	4/6	(66.6%)	3/5	(60%)	13/36	36.1
Total (wt only)		3/28	(10.7%)	5/17	(29.4%)	3/20	(15%)	5/11	(45.4%)	3/5	(60%)	19/81	23.4

NA not available

Statistical analysis+ of the data of the recovery of ts mutants from the footpad.

(A) Comparison of mouse strains

Group	Group	Int. Est.	
1	2	-2.8192	0.1971
1	3	-2.7371	0.2236
1	4	-3.7102	-0.8913*
1	5	-3.4760	-0.6525*
2	3	-1.0332	1.1418
2	4	-1.9784	-0.0009*
2	5	-1.7452	0.2389
3	4	-1.9899	-0.0981*
3	5	-1.7568	0.1418
4	5	-0.5979	1.0709

Estimated Probabilities

0.0258 0.0963 0.0920 0.2266 0.1875

Where group 1 = PIRBRIGHT
2 = C57BL/6-J
3 = BIOZZI
4 = BALB/c
5 = A/MICE

0.025 0.029 0.09 0.18 0.22

1 3 2 5 4

Conclusion

Group 4 (BALB/c) is definitely greater in proportion of +ve than group 1, 2 and 3. Also group 5 is greater than group 1.

+ See Discussion Table 1 for details of the method of the statistical analysis

* significant difference

continued/.....

(B) Comparison of ts viruses

Group	Group	Int. Est.	
1	2	-2.1047	1.5903
1	3	-2.5623	0.7987
1	4	-2.2027	1.1856
1	5	-0.8492	6.6741
1	6	-1.4937	2.3502
1	7	-1.4579	2.7491
1	8	-2.1612	1.6467
1	9	-1.9280	1.8438
1	10	-2.2871	1.2147
1	11	-0.8670	6.6581
1	12	-1.8654	2.0239
1	13	-1.6238	2.6038
2	3	-2.2655	1.0163
2	4	-1.9061	1.4035
2	5	-0.5744	6.9138
2	6	-1.2020	2.5728
2	7	-1.1691	2.9748
2	8	-1.8691	1.8691
2	9	-1.6356	2.0658
2	10	-1.9919	1.4339
2	11	-0.5923	6.8978
2	12	-1.5740	2.2469
2	13	-1.3352	2.8296
3	4	-1.0928	1.8393
3	5	0.1297	7.4588*
3	6	-0.4143	3.0343
3	7	-0.3971	3.4520
3	8	-1.0796	2.3288
3	9	-0.8443	2.5237
3	10	-1.1858	1.8769
3	11	0.1118	7.4428*
3	12	-0.7885	2.7106
3	13	-0.5640	3.3076
4	5	-0.2498	7.0918
4	6	-0.8008	2.6743
4	7	-0.7823	3.0907
4	8	-1.4663	1.9690
4	9	-1.2312	2.1640
4	10	-1.5739	1.5185
4	11	-0.2677	7.0758
4	12	-1.1748	2.3504
4	13	-0.9491	2.9461
5	6	-6.2656	1.2971
5	7	-6.1436	1.6100
5	8	-6.9420	0.6026
5	9	-6.7178	0.8086
5	10	-7.1461	0.2487
5	11	-4.9931	4.9592
5	12	-6.6262	0.9597
5	13	-6.3049	1.4599
6	7	-1.9212	2.3561
6	8	-2.6281	1.2573
6	9	-2.3953	1.4547
6	10	-2.7574	0.8285
6	11	-1.3150	6.2496
6	12	-2.3315	1.6336
6	13	-2.0869	2.2105

7	8	-3.0254	1.2196
7	9	-2.7941	1.4185
7	10	-3.1682	0.8044
7	11	-1.6278	6.1276
7	12	-2.7254	1.4926
7	13	-2.4682	2.1569
8	9	-1.6919	2.1222
8	10	-2.0527	1.6946
8	11	-0.6205	6.9259
8	12	-1.6287	2.3016
8	13	-1.3854	2.8799
9	10	-2.2484	1.2601
9	11	-0.8265	6.7017
9	12	-1.8263	2.0690
9	13	-1.5844	2.6486
10	11	-0.2666	7.1301
10	12	-1.2017	2.4327
10	13	-0.9710	3.0234
11	12	-6.6101	0.9776
11	13	-6.2889	1.4778
12	13	-1.7583	2.5798

continued/...

Estimated Probabilities

0.1273	0.1600	0.2679	0.2000	0.0000	0.0857	
0.0678	0.1591	0.1321	0.2037	0.0000	0.1176	0.0784

Where group 1-13 represents the ts mutants viruses (ts1-ts13).

* significant difference

0.0	0.0	0.07	0.08	0.09	0.12	0.13	0.13	0.16	0.16	0.20	0.20	0.27
ts 5	ts 11	ts 7	ts 13	ts 6	ts 12	ts 1	ts 9	ts 8	ts 2	ts 4	ts 10	ts 3

The wild type estimated probability = 0.24

Conclusion

Only significant difference is that ts 3 has high probability of positive than ts 5 and ts 11.

N.B

Statistical studies of the data was carried out in the department of statistics/Glasgow University

DISCUSSION TABLE 3

ANALYSIS OF THE DATA ACCORDING TO THE SITE OF REACTIVATION

Virus	pfu/ mouse	No. of mice•	PIRBRIGHT				No. of mice•	C57BL/6-J				No. of mice•	BIOZZI			
			(FP & DRG)$^-$	(FP)$^+$	(DRG)$^+$	(FP & DRG)$^+$		(FP & DRG)$^-$	(FP)$^+$	(DRG)$^+$	(FP & DRG)$^+$		(FP & DRG)$^-$	(FP)$^+$	(DRG)$^+$	(FP & DRG)$^+$
ts1	$1x10^5$	11	10	0	1	0	10	6	0	4	0	9	4	2	3	0
ts2	$1.7x10^6$	11	10	1	0	0	8	6	2	0	0	16	7	1	8	0
ts3	$1.2x10^5$	5	4	1	0	0	9	7	1	1	0	15	10	5	0	0
ts4	$5x10^6$	15	13	0	2	0	13	8	1	4	0	14	2	0	11	1
ts5	$1.7x10^6$	13	13	0	0	0	12	11	0	1	0	12	12	0	0	0
ts6	$1x10^6$	14	13	0	1	0	12	9	1	2	0	15	14	1	0	0
ts7	$8x10^5$	15	9	0	6	0	9	8	1	0	0	9	7	0	1	1
ts8	$2.5x10^5$	11	11	0	0	0	5	3	0	1	1	9	6	0	2	1
ts9	$7.5x10^5$	13	13	0	0	0	13	10	3	0	0	10	8	2	0	0
ts10	$2.5x10^5$	11	11	0	0	0	12	12	0	0	0	9	9	0	0	0
ts11	$4.2x10^5$	12	12	0	0	0	12	12	0	0	0	12	11	0	1	0
ts12	$5x10^5$	12	8	1	3	0	7	3	1	2	1	14	13	1	0	0
ts13	$6x10^5$	9	6	1	2	0	7	7	0	0	0	14	12	0	2	0
Total (mutants)		152	133	4	15	0	129	102	10	15	2	158	115	12	28	3
wt	$1x10^5$	12	5	0	7	0	4	0	1	3	0	11	1	0	9	1
	$1x10^6$	5	0	0	5	0	11	2	3	5	1	6	0	0	4	2
Total (wt only)		17	5	0	12	0	15	2	4	8	1	17	1	0	13	3

Virus	pfu/ mouse	No. of mice•	BALB/c				No. of mice•	A			
			(FP & DRG)$^-$	(FP)$^+$	(DRG)$^+$	(FP & DRG)$^+$		(FP & DRG)$^-$	(FP)$^+$	(DRG)$^+$	(FP & DRG)$^+$
ts1	$1x10^5$	11	3	2	3	3	10	5	0	5	0
ts2	$1.7x10^6$	6	4	2	0	0	9	1	0	6	2
ts3	$1.2x10^5$	13	10	3	0	0	12	7	5	0	0
ts4	$5x10^6$	9	3	2	3	1	10	2	2	0	6
ts5	$1.7x10^6$	11	11	0	0	0	11	11	0	0	0
ts6	$1x10^6$	15	13	1*	1	0	10	5	3	2	0
ts7	$8x10^5$	10	3	2	5	0	11	11	0	0	0
ts8	$2.5x10^5$	9	1	0	4	4	8	1	0	6	1
ts9	$7.5x10^5$	9	7	2	0	0	8	7	0	1	0
ts10	$2.5x10^5$	9	1	4	2	2	10	3	5	2	0
ts11	$4.2x10^5$	10	6	0	4	0	11	10	0	1	0
ts12	$5x10^5$	3	2	0	1	0	11	7	1	3	0
ts13	$6x10^5$	10	9	1	0	0	11	8	2	1	0
Total (mutants)		125	73	19	23	10	132	78	18	27	9
wt	$1x10^5$	3	1	0	2	0	NC				
	$1x10^6$	6	0	0	2	4	5	0	0	2	3
Total (wt only)		9	1	0	4	4	5	0	0	2	3

* Not isolated

** No. of positive mice = total no. of mice - (FP and DRG)$^{-ve}$

NC Not considered due to loss of cultured FP by contamination

• Only cultures of footpad and ganglia, from a single mouse, that proved to be clean for more than 3 weeks after explantation were considered in this analysis

/for Summary see next page

DISCUSSION TABLE 3 CUMULATIVE DATA

Virus	pfu/ mouse	No. of mice•	CUMULATIVE DATA			
			(FP AND DRG)$^-$	(FP)$^+$/no of +ve mice**	(DRG)$^+$/no Of +ve mice	(FP AND DRG)$^+$ /no of +ve mice
ts1	$1x10^5$	51	28	4/23 (17.3%)	16/23 (69.5%)	3/23 (13%)
ts2	$1.7x10^6$	50	28	6/22 (27.2%)	14/22 (63.6%)	2/22 (9%)
ts3	$1.2x10^5$	54	38	15/16 (93.7%)	1/16 (6.2%)	0/16 (0%)
ts4	$5x10^6$	61	28	5/33 (15.1%)	20/33 (60.6%)	8/33 (24.2%)
ts5	$1.7x10^6$	59	58	0/1 (0%)	1/1 (100%)	0/1 (0%)
ts6	$1x10^6$	66	54	6/12 (50%)	6/12 (50%)	0/12 (0%)
ts7	$8x10^5$	54	38	3/16 (18.7%)	12/16 (75%0	1/16 (6.2%)
ts8	$2.5x10^5$	42	22	0/20 (0%)	13/20 (65%)	7/20 (35%)
ts9	$7.5x10^5$	53	45	7/8 (87.5%)	1/8 (12.5%)	0/8 (0%)
ts10	$2.5x10^5$	51	36	9/15 (60%)	4/15 (26.6%)	2/15 (13.3%)
ts11	$4.2x10^5$	57	51	0/6 (0%)	6/6 (100%)	0/6 (0%)
ts12	$5x10^5$	47	33	4/14 (28.5%)	9/14 (64.2%)	1/14 (7.1%)
ts13	$6x10^5$	51	42	4/9 (44.4%)	5/9 (55.5%)	0/9 (0%)
Total (mutants)		696	501	63/195 (32.2%)	108/195 (55.3%)	24/195 (12.3%)
wt	$1x10^5$	30	7	1/23 (4.3%)	21/23 (91.3%)	1/23 (4.3%)
	$1x10^6$	33	2	3/31 (9.6%)	18/31 (58%)	10/31 (32.2%)
Total (wt only)		63	9	4/54 (7.4%)	39/54 (72.2%)	11/54 (20.3%)

DISCUSSION TABLE 4

DISTRIBUTION OF RECOVERABLE VIRUSES IN THE DRG OF MICE*

Virus	pfu/ mouse	Mice strain	No of mice (releasing virus)	DRG: Sacral 1st	Lumbar 6	Lumbar 5	Lumbar 4	Lumbar 3	Lumbar 2	Lumbar 1	Thoracic 13th	Thoracic 12th	Left 1	side 2
ts1	$1x10^5$	PIRBRIGHT	1	0	1	0	0	0	0	0	0	0	0	0
ts4	$5x10^6$		2	0	1	1	0	0	0	0	0	0	0	0
ts6	$1x10^6$		1	0	0	1	0	0	0	0	0	0	0	0
ts7	$8x10^5$		6	0	4	2	0	0	0	0	0	0	0	0
ts8	$2.5x10^5$		1	0	0	1	0	0	0	0	0	0	0	0
ts12	$5x10^5$		3	0	1	2	0	0	0	0	0	0	0	0
ts13	$6x10^5$		2	0	1	1	0	0	0	0	0	0	0	0
wt	$1x10^6$		6	4	7	8	5	5	2	2	3	1	0	0
wt	$1x10^5$		9	1	5	6	3	2	2	2	0	0	0	0
Total (mutants)			16	0	8	8	0	0	0	0	0	0	0	0
Total (wt only)			15	5	12	14	8	7	4	4	3	1	0	0
ts1	$1x10^5$	C57BL/6-J	5	1	1	3	2	0	0	0	0	0	0	0
ts3	$1.2x10^5$		1	0	1	1	0	0	0	0	0	0	0	0
ts4	$5x10^6$		4	1	1	2	3	0	0	0	0	0	0	0
ts5	$1.7x10^6$		1	0	0	1	0	0	0	0	0	0	0	0
ts6	$1x10^6$		2	0	1	1	0	0	0	0	0	0	0	0
ts8	$2.5x10^5$		4	0	1	2	1	0	0	0	0	0	0	0
ts11	$4.2x10^5$		1	0	0	1	0	0	0	0	0	0	0	0
ts12	$5x10^5$		5	2	2	3	0	0	0	0	0	0	0	0
wt	$1x10^5$		4	2	2	0	1	0	0	0	0	0	0	0
wt	$1x10^6$		8	1	5	6	4	0	0	0	0	0	0	0
Total (mutants)			23	4	7	14	6	0	0	0	0	0	0	0
Total (wt only)			12	3	7	6	5	0	0	0	0	0	0	0
ts1	$1x10^5$	BIOZZI	4	0	1	2	1	0	0	0	0	0	0	0
ts2	$1.7x10^6$		8	2	4	3	2	0	0	0	0	0	0	0
ts4	$5x10^6$		12	2	5	6	4	0	0	0	0	0	0	0
ts6	$1x10^6$		1	0	0	0	1	0	0	0	0	0	0	0
ts7	$8x10^5$		2	0	0	2	2	0	0	0	0	0	0	0
ts8	$2.5x10^5$		5	0	3	2	0	0	0	0	0	0	0	0
ts11	$4.2x10^5$		1	0	1	0	0	0	0	0	0	0	0	0
ts13	$6x10^5$		2	0	2	1	0	0	0	0	0	0	0	0
wt	$1x10^5$		11	1	1	5	6	2	1	0	0	0	0	0
wt	$1x10^6$		6	0	3	5	3	0	0	0	0	0	0	0
Total (mutants)			35	4	16	16	10	0	0	0	0	0	6	6
Total (wt only)			17	1	4	10	9	2	1	0	0	0	0	0
ts1	$1x10^5$	BALB/c	8	1	2	6	4	2	0	0	0	0	0	0
ts2	$1.7x10^6$		1	0	1	0	0	0	0	0	0	0	0	0
ts4	$5x10^6$		11	1	3	7	3	1	0	0	0	0	0	0
ts6	$1x10^6$		1	0	0	1	0	0	0	0	0	0	0	0
ts7	$8x10^5$		5	0	3	5	1	0	0	0	0	0	0	0
ts8	$2.5x10^5$		8	0	4	7	1	0	0	0	0	0	0	0
ts10	$2.5x10^5$		4	0	3	1	2	0	0	0	0	0	0	0
ts11	$4.2x10^5$		4	1	3	1	1	0	0	0	0	0	0	0
ts12	$5x10^5$		5	1	2	3	0	0	0	0	0	0	0	0
wt	$1x10^5$		2	0	2	1	1	0	0	0	0	0	0	0
wt	$1x10^6$		6	1	4	6	4	3	0	0	0	0	0	0
Total (mutants)			47	4	21	31	12	3	0	0	0	0	0	0
Total (wt only)			8	1	6	7	5	3	0	0	0	0	0	0
ts1	$1x10^5$	A/mice	7	0	4	5	1	0	0	0	0	0	0	0
ts2	$1.7x10^6$		8	0	1	7	5	1	0	0	0	0	0	0
ts4	$5x10^6$		6	0	3	4	2	2	0	0	0	0	0	0
ts6	$1x10^6$		2	0	1	1	0	0	0	0	0	0	0	0
ts8	$2.5x10^5$		7	0	4	7	2	0	0	0	0	0	0	0
ts9	$7.5x10^5$		1	0	0	1	0	0	0	0	0	0	0	0
ts10	$2.5x10^5$		2	0	1	0	1	0	0	0	0	0	0	0
ts11	$4.2x10^5$		2	0	0	1	1	0	0	0	0	0	0	0
ts12	$5x10^5$		4	0	1	3	0	0	0	0	0	0	0	0
ts13	$6x10^5$		1	0	1	1	0	0	0	0	0	0	0	0
wt	$1x10^5$		4	0	3	2	3	2	2	2	2	0	0	0
wt	$1x10^6$		5	1	5	5	3	1	3	0	3	3	0	0
Total (mutants)			40	0	16	30	12	3	0	0	0	0	0	0
Total (wt only)			9	1	8	7	6	3	5	2	2	0	0	0

* Only those mutants that were recoverable from the DRG are listed.
+ A single mouse may shed virus in more than one ganglion.

DISCUSSION TABLE 5

TIME OF VIRUS REACTIVATION FROM FP AND DRG IN DIFFERENT STRAINS OF MICE

Virus	pfu/ mouse	Mice Strains									
		PIRBRIGHT		C57BL/J		BIOZZI		BALB/c		A	
		DRG	FP	DRG	FP	DRG	FP	DRG	FP	DRG	FP
ts1	$1x10^5$	16	-	11-21 (18.2)	-	14-17 (15.2)	14-21 (17.5)	15-23 (18.8)	17-32 (24.8)	11-20 (15.8)	-
ts2	$1.7x10^6$	-	44	-	28.5	17-23 (19.2)	23	23	24	14-16 (15.2)	17-21 (19)
ts3	$1.2x10^5$	-	34	28	36	-	10-25 (15.8)	-	11-20 (14)	-	14-16 (14.6)
ts4	$5x10^6$	20	-	10-20 (17)	14	10-20 (16.2)	37	14-18 (17)	18-27 (20.7)	11-16 (13.1)	12-19 (15.8)
ts5	$1.7x10^6$	-	-	23	-	-	-	-	-	-	-
ts6	$1x10^6$	17	-	19-23 (21)	16	19	30	13	19	13-28 (20.5)	20-24 (22.6)
ts7	$8x10^5$	16-22 (17)	-	-	23	16-22 (19)	37	-	14	-	-
ts8	$2.5x10^5$	21	-	8-15 (12.3)	15	11-19 (14.8)	14	11-14 (11.3)	12-22 (16)	14	18
ts9	$7.5x10^5$	-	-	-	19-25 (22.3)	-	12-16 (14)	-	23	25	-
ts10	$2.5x10^5$	-	-	-	-	-	-	12-19 (17.2)	15-26 (21.6)	19	13-45 (20.2)
ts11	$4.2x10^5$	-	-	14	-	14	-	17-25 (19.2)	-	14-25 (14.5)	-
ts12	$5x10^5$	17	8	17-18 (17.4)	8-21 (15.3)	-	23	14	-	10-16 (14)	15
ts13	$6x10^5$	14-18 (16)	20	-	-	11-14 (12.5)	-	-	18	18-24 (22)	13-18 (15.5)
wt	$1x10^5$	8-26 (15.8)	18-24 (22)	10-19 (15.5)	23	10-26 (21.1)	43	18	17	7-28 (16.7)	-
wt	$1x10^6$	10-13 (11.5)	-	9-16 (12.7)	13-19 (15.7)	10-12 (11.3)	13-17 (15)	7-8* (7.6)	8-12* (9.7)	10-15 (11)	10-27 (15.6)

* Ganglia and footpads were cultured and screened at 38.5°C
x - x earliest and latest time of virus recovery
\- no virus recovered
( ) average time of reactivation

Single numbers indicate that virus was recovered in one occasion only.

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Viral Genes Modify Herpes Simplex Virus Latency both in Mouse Footpad and Sensory Ganglia

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SUMMARY

Herpes simplex virus type 2 wild-type and 13 temperature-sensitive mutants have been examined for their ability to be recovered from latently infected Biozzi mice. After inoculation into the rear footpad, virus could be recovered from both the dorsal root ganglia (DRG) and the footpad (FP) at the site of inoculation (wt, *ts* 1, *ts* 2, *ts* 4, *ts* 6, *ts* 7 and *ts* 8), or from the DRG only (*ts* 11 and *ts* 13), or from FP only (*ts* 3, *ts* 9, *ts* 12). Two mutants (*ts* 5 and *ts* 10) have not been recovered from either site. Both DNA-positive and DNA-negative mutants have been shown to be capable of establishing latent infection.

Following a primary infection in man, herpes simplex virus (HSV) usually establishes latency, from which it may periodically reactivate and release virus; this can result in overt clinical disease. HSV genetic information persists in sensory ganglia both in man and in experimental animals, and after explantation of ganglia followed by culture *in vitro* the latent virus can be reactivated (Stevens & Cook, 1971; Stevens, 1975; Bastian *et al.*, 1972; Barringer, 1975).

HSV-1 temperature-sensitive (*ts*) mutants have been used to probe the mechanism of latency, and differences in ability to be recovered from latency have been described (Lofgren *et al.*, 1977; Watson *et al.*, 1980; Clements & Subak-Sharpe, 1983). In this paper we describe for the first time the ability of HSV-2 *ts* mutants to be recovered from the latent state in mouse dorsal root ganglia (DRG). In addition, we have been able to recover HSV-2 from another tissue, the footpad (FP) at the site of inoculation 3 months or more after infection.

HSV-2 wild-type (wt) virus and a set of 13 *ts* mutants were used (Timbury, 1971; Halliburton & Timbury, 1973, 1976) to inoculate 3- to 4-week-old Biozzi high antibody responder strain mice of both sexes via the rear FP (10^5 to 10^6 p.f.u.). No significant difference in response between males and females was found. It is relevant that the mouse body core temperature (38·5 °C) is non-permissive for the *ts* mutants used. The FP may be somewhat below the core temperature when the mice are active; however, when they are closely packed asleep in the nest, the FP temperature is probably at, or very close to, that of the core. No animal died or became paralysed following infection of Biozzi mice with the HSV-2 wt and *ts* mutants at the doses indicated in Table 1. Some mice showed slight FP swelling immediately after the initial inoculation, but no swelling or symptoms of active infection were noted subsequently.

At least 3 months after primary infection (when the mice were asymptomatic), the animals were killed using chloroform. Nine ipsilateral (one sacral, six lumbar, two thoracic) and two contralateral DRG were rapidly dissected out and explanted under aseptic conditions. In addition, FP tissue from both the left and the right rear limbs was also explanted from each mouse and grown as organ cultures *in vitro* at 31 °C. In 16 cases out of a total of 199, the explanted FP became contaminated during culture and had to be discarded. Explant cultures of individual ganglia and FP tissue were maintained in 150 µl Eagle's minimum essential medium (Glasgow modified) supplemented with 50% foetal calf serum, penicillin (100 units/ml), streptomycin (100 µg/ml), gentamicin (25 µg/ml) and nystatin (25 units/ml) in microtitre plates. Released virus was

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Table 1. *Recovery of wild-type HSV and* ts *mutants following explantation of dorsal root ganglia (DRG) and footpad (FP) from latently infected mice*

Input dose (p.f.u./mouse)	Virus (10^5–10^6 p.f.u./mouse)	DNA phenotype	DRG positive/ total (%)	FP positive/ total (%)
1×10^6	*wt* HG52	+	6/6 (100)	2/6 (33)
1×10^5	*wt* HG52	+	11/13 (85)	1/14 (7)
1×10^5	*ts* 1	−	4/11 (36)	2/10 (20)
$1{\cdot}7 \times 10^6$	*ts* 2	−	8/17 (47)	1/16 (6)
$1{\cdot}2 \times 10^5$	*ts* 3	+	0/16 (0)	5/15 (33)
5×10^6	*ts* 4	+	12/14 (86)	1/14 (7)
$1{\cdot}7 \times 10^6$	*ts* 5	+	0/12 (0)	0/12 (0)
1×10^6	*ts* 6	−	1/16 (6)	1/16 (6)
8×10^5	*ts* 7	−	2/14 (14)	1/11 (9)
$2{\cdot}5 \times 10^5$	*ts* 8	−	5/14 (35)	1/9 (11)
$7{\cdot}5 \times 10^5$	*ts* 9	−	0/11 (0)	2/11 (18)
$2{\cdot}5 \times 10^5$	*ts* 10	−	0/13 (0)	0/9 (0)
$4{\cdot}2 \times 10^5$	*ts* 11	−	1/12 (8)	0/12 (0)
5×10^5	*ts* 12	+	0/14 (0)	1/14 (7)
6×10^5	*ts* 13	+	2/14 (14)	0/14 (0)

detected by screening the supernatant on semi-confluent BHK cells grown in microtitre plates. On several occasions, an FP was divided into two: one half was incubated as above, and the other homogenized and immediately screened. At no time was virus found in the homogenate, although virus reactivated from the cultivated half-FP in several cases. The screening procedure was carried out daily for the explanted FP cultures and twice weekly for DRG. Recovered viruses were checked for growth on BHK C13 cells both at 31 °C and at 38·5 °C (the permissive and non-permissive temperatures). A mouse in which either the FP and/or any ganglion released virus on one or more occasions was scored as positive. The first screening always took place within 24 h after explantation.

As no mice died after inoculation of 10^5 p.f.u. of HSV-2 strain HG52 wt virus, the Biozzi high antibody responder strain falls into the group of mouse strains relatively resistant to acute infection with HSV to which, for example, C57BL/6 mice also belong (Lopez, 1975; Kirchner *et al.*, 1978; Clements & Subak-Sharpe, 1983). Details of comparative work using HSV-1 and HSV-2 with five mouse strains will be published in due course.

Virus was never detected in the supernatant from explanted DRG earlier than the 6th day after dissection. The majority of HSV-2 reactivants from explanted ganglia first appeared between day 7 and 14 and the latest time at which virus was first detected was 24 days after DRG explantation. To our surprise, we reproducibly obtained virus reactivation also from FP explants of mice infected with HSV-2. The FP cultures never shed virus before the 10th day after explantation and on one occasion not before 42 days after explantation. No virus has ever been detected in tissue homogenates immediately after explantation, which is in keeping with other reports (Cook & Stevens, 1976; Hill & Blyth, 1976). At the doses of HSV used in the present study, virus was never recovered from the left DRG or the left FP, contralateral to the site of inoculation. The virus that reactivated from mice initially infected with a *ts* mutant was checked and in every case proven to have retained the *ts* phenotype. Using the restriction enzymes *Bam*HI, *Bgl*II, *Hin*dIII, *Kpn*I and *Eco*RI (Lonsdale, 1979), the DNA fragment profiles of reactivated viruses were compared to the profiles of the virus used for initial infection. In every case the profiles were shown to be unchanged.

As can be seen from Table 1, HSV-2 wt virus was recovered with high efficiency from the DRG but only infrequently from the FP; the efficiency of recovery was dose-dependent. Two HSV-2 mutants, *ts* 5 and *ts* 10, have not been recovered following explantation either from DRG or from the FP; mutants *ts* 3, *ts* 9 and *ts* 12 have only been recovered from explanted FP, while mutants *ts* 11 and *ts* 13 have been recovered from DRG only. The remaining HSV-2 mutants were all recoverable both from DRG and FP, but whilst *ts* 1, *ts* 6 and *ts* 7 were obtained from either site with approximately equal frequency, *ts* 2, *ts* 4 and *ts* 8 were much more frequently reactivated from the DRG.

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Table 2. *Anatomical distribution of DRG from which HSV-2 was successfully reactivated in latently infected Biozzi mice**

			Right side DRG‡							
			Lumbar						Thoracic	
Virus†	No. of mice	Sacral 1	6	5	4	3	2	1	13	12
wt	13	1	1	5	6	2	1	0	0	0
ts 1	11	0	1	2	1	0	0	0	0	0
ts 2	17	2	4	3	2	0	0	0	0	0
ts 4	14	2	5	6	4	0	0	0	0	0
ts 6	18	0	0	0	1	0	0	0	0	0
ts 7	14	0	0	2	2	0	0	0	0	0
ts 8	14	0	3	2	0	0	0	0	0	0
ts 11	12	0	1	0	0	0	0	0	0	0
ts 13	14	0	2	1	0	0	0	0	0	0

* The figures under each column indicate the number of mice positive with respect to virus recovery from that DRG.

† Mutants *ts* 3 (16 mice), *ts* 5 (12), *ts* 9 (11), *ts* 10 (13) and *ts* 12 (14) have not been recovered from DRG.

‡ No virus was ever recovered from the left side DRG.

The distribution of ganglia from which HSV-2 was recovered was not the same for all the latency-positive mutants. Mutants *ts* 2 and *ts* 4 were, like wt, recovered from a number of ganglia, some of which would not be expected to innervate the footpad directly, whereas the other mutants were shed from only one or two of the ganglia innervating the footpad (Table 2).

The above results suggest the following tentative conclusions. (i) Non-recovery of HSV-2 mutants *ts* 5 and *ts* 10 from either site suggests that these mutants are deficient in functions either necessary for the establishment or maintenance of latency in Biozzi mice, or for the ability to reactivate upon explantation. (ii) The recovery of some HSV-2 mutants (*ts* 3 and also *ts* 9 and *ts* 12) only from the explanted FP suggests that HSV-2 can become latent in tissues other than, and apparently independent of, the DRG. (iii) The recovery of the HSV-2 mutants *ts* 11 and *ts* 13 only from DRG supports the notion of independent latency in FP and DRG. (iv) Only four mice produced virus both from the FP and from DRG following explantation, one infected with HSV-2 wt and one each with *ts* 4, *ts* 7 and *ts* 8. This is in keeping with the hypothesis that HSV-2 can produce latent infection independently at the two sites. (v) The observed differences in reactivation of HSV-2 *ts* mutants from DRG and/or FP, and the apparent differences between *ts* mutants in recoverability, are evidence that the genetic control of the ability of HSV to produce latent infection is not simple and at least in part due to several virus gene functions. It is relevant in this context that both DNA-positive and DNA-negative mutants were shown to induce latent infection.

All the reactivants had retained the *ts* phenotype which excludes reversion to the wt, temperature-resistant phenotype as an explanation for the recovery of some of the *ts* mutants. It is not possible to exclude that some leakiness allowing replication may have occurred, particularly in the mouse FP which may not always have been at the mouse core temperature. Any leak-through would tend to increase the capacity for viral gene expression and therefore favour the establishment of latency. These considerations can not help to explain the non-recovery of mutants *ts* 5 and *ts* 10.

There is good evidence that the viral genome is latent within the sensory neurones of DRG (McLennan & Darby, 1980), but recovery from central nervous tissue, autonomic ganglia and the adrenal gland has also been reported (Cook & Stevens, 1976; Nesburn *et al.*, 1972; Warren *et al.*, 1978; Price *et al.*, 1975; Knotts *et al.*, 1973). Skin explant cultures from the sites in man at which HSV recurrences had been observed did not yield virus (Rustigan *et al.*, 1966). However, HSV has been shown to be present in peripheral tissues of latently infected animals under some circumstances. Scriba (1976, 1977) isolated HSV-1 and HSV-2 from the site of inoculation of latently infected guinea-pigs (FP and vagina). Donnenberg *et al.* (1980) have also isolated HSV-2

from guinea-pig FP. Hill *et al.* (1980) reported the isolation of HSV-1 from the ear skin (the site of inoculation) in 8% of latently infected mice in the absence of clinical lesions. Recurrent HSV in patients with 'blow-out' fractures, which had severed the nerve supply to the area of skin involved, has also been observed (Hoyt & Billson, 1976). Scriba (1981) recovered latent HSV-2 from FP skin, but not the DRG, of guinea-pigs infected with virus into the FP 10 days after surgical denervation. Recovery of virus from the peripheral regions may be due either to reactivation of virus latent within nervous tissue which subsequently travels via a nerve to the peripheral site, or to the presence of virus over a long period at peripheral sites, although these two possibilities are not mutually exclusive. We think it most unlikely that the input virus could persist either as such or in a slowly replicating form during the 3 months or more elapsing between inoculation and dissection. We suggest that the virus is capable of establishing a latent infection in the FP and, as neural cell bodies have never been described as located in the mouse FP, it follows that the latency is established in cells other than neurones. The observed difference in timing of reactivation from the FP and DRG may be a result of this. At present, we cannot exclude the possibility of mechanical trapping after reactivation but before release of virus from the FP, contributing to the observed difference in timing of virus release. We feel this alone is unlikely to lead to such a large and consistent difference as is observed. We are presently extending the study using immunofluorescence and *in situ* hybridization.

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