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Comparative Studies of Herpes Simplex Virus Types 1 and 2

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**COMPARATIVE STUDIES OF HERPES
SIMPLEX VIRUS TYPES 1 AND 2**

by

Joseph Lloyd Waner

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**A Dissertation Submitted to the Faculty of the Graduate
School of Loyola University in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy**

June

1969

LIFE

Joseph Lloyd Waner was born in Detroit, Michigan, February 4, 1942.

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CHAPTER I

INTRODUCTION

The clinical appearance of herpetic infections has long been recognized by physicians. A Roman physician, around 100 A.D., described herpetic eruptions around the mouth during periods of fever (Mettler, 1947). Today eruptions around the mouth are commonly known as fever blisters or cold sores. Lesions of similar appearance may appear anywhere on the body, eyes, face, arms, thighs or genitally. Indeed, the word "herpes" in Greek means "to creep" and was used for centuries to describe spreading cutaneous lesions of varied etiology (Cumston, 1926). In man, when first infected, a primary lesion occurs which recedes with the appearance of a rise in specific neutralizing antibody. The viral agent, however, becomes latent in the host and may cause periodic eruptions with the same clinical appearance as the primary lesion. Occasionally, a herpetic encephalitis occurs in individuals whose immune defenses have been suppressed by drugs or other debilitating factors.

Herpes labialis, was recognized clinically for at least

two millennia before Astruc (1736) described herpes genitalis in the eighteenth century. Investigation of the etiological agent, or agents, was delayed until Grutar (1924) provided the first tool for the study of herpesviruses. In 1920 he succeeded in transmitting herpetic ocular virus from man to the rabbit cornea. The agent was soon demonstrated to be filterable (Lugar and Lauda, 1921) and to cause encephalitis in rabbits (Doerr and Schnabel, 1921). Using this information, Lipschutz (1921) conducted the first comparative study of genital and non-genital herpes isolates. He inoculated the rabbit cornea with genital and non-genital isolates and observed that they differed in their cytopathological effects. He noted that a cornea infected with material from herpes labialis could not be reinfected with similar material. It could, however, be reinfected with material from herpes genitalis. This indicated an immune specificity. He also reported that cases of venereal herpes were sometimes accompanied by neurological complaints and that herpetic eye involvements, though often associated with herpes labialis, were never associated with genital herpes. Overall, Lipschutz maintained that herpes labialis and herpes genitalis were biologically related, but etiologically different.

Since Lipschutz's early observations many attempts have been made over the years to detect antigenic differences between strains of herpes simplex virus. The strains isolated,

however, were usually from cases of herpes labialis. Egg and mouse neutralization tests were commonly employed because tissue culture systems were not available. Such systems introduced variables that could make small antigenic differences undetectable. Using these systems and examining strains isolated from the same clinical syndrome, numerous workers reported no antigenic differences between strains of herpes simplex (Burnet and Lush, 1939; Hayward, 1950; Kilbourne and Horsfall, 1951; Carabedian and Syverton, 1955).

The first evidence suggesting antigenic differences between herpes isolates resulted from a comparison of an isolate from a woman with primary herpetic vulvovaginitis with an established laboratory strain originally isolated from herpes labialis (Slavin and Gavett, 1946). Using undiluted patient sera and the mouse neutralization test, a considerable difference was detected. Antisera from patients suffering from cold sores gave little or no protection when the challenge virus was the genital isolate. Neither did anti-serum prepared in rabbits against the genital isolate show protection against the virus isolated orally.

Jawetz (1955) employed neutralization tests using eggs and mice in a study of isolates from various sources. He found that a strain isolated from a recurrent thigh lesion consistently differed with isolates from the wrist, brain, and a primary

herpetic infection of the throat. It has recently been reported that herpetic thigh lesions are usually associated with herpes genitalis (Dowdle, et al., 1967).

In 1962 the four strains isolated by Jawetz (1955) along with 27 others were examined by Schneeweis (1962). He employed neutralization tests using plaque reduction in HeLa cells, constant doses of virus against varying dilutions of sera, and neutralization kinetic studies. His tests revealed that the strains could be divided into two types. Type 1 consisted of isolates from clinical cases of keratoconjunctivitis, dermatitis, labialis, encephalitis and eczema herpeticum. All strains classified as type 2 were from herpetic penile lesions. The serological difference between the types could only be demonstrated consistently using antiserum to type 1 strains.

Shubladze (1959) examined eleven herpes isolates and divided them into three antigenically distinct groups. One of these groups, however, has been shown to be represented by a strain of virus resembling Japanese B encephalitis virus (Flummer, 1964).

Flummer (1964) confirmed the findings of Shubladze (1959) that two of the strains, "L2" and "US", were antigenically different types. He also found that the "KS" strain of Gudnadottir (1964) resembled the "US" strain. By using cross neutralization curves, he could differentiate the two types

with antisera to either type. This finding differed from that of Schneeweis (1962) who could only detect differences with antisera to non-genital strains. Pauls and Dowdle (1967) also found that "L2" represented one antigenic group and "MS" and "US" a second group. Their results more closely agreed with Schneeweis (1962) in that the antigenic differences were essentially one way and more easily observed with antiserum to strain "L2". Implementing a microneutralization test in the study of over one hundred different isolates, this same group confirmed Schneeweis' earlier observation that the two antigenic types were associated with the site of viral isolation. Type 1 was shown to be primarily recovered from non-genital areas while type 2 was recovered from genital sites. Strain "L2" was characteristic of a type 1 isolate whereas strains "US" and "MS" were associated with genital isolates (Dowdle et al., 1967).

Since the first report of transmission from man to the rabbit cornea of herpes simplex (Grutar, 1924), studies have been undertaken in animals to determine herpesvirus pathogenesis. Goodpasture and Teague (1923) reported that herpesvirus hominis could be transmitted from a peripheral form of infection directly to the central nervous system along sensory, motor, or sympathetic nerve fibers, depending on the route of inoculation. These same workers noticed differences in the

neurotropism of various herpes strains in the rabbit. On corneal inoculation of two strains originating from herpes labialis, one strain induced keratoconjunctivitis with no encephalitis and the other caused both keratoconjunctivitis and encephalitis with subsequent death. Other studies (Levaditi, 1926) reported 13% to 100% mortality following corneal inoculation with a variety of strains. It must be pointed out, however, that these investigators paid no attention to the passage level in rabbits of the strains investigated. Early work on rabbits showed that multiple passages increased the virulence of herpesvirus for that animal (Grutar, 1924).

Rapp (1963), when studying the effect of laboratory variants of herpesvirus, found that, in general, small plaque variants were less capable of promoting keratitis than large plaque variants. The first virulence comparison of a type 1 versus a type 2 strain was unknowingly done by Wheeler (1960). A variant he had isolated, and which was later shown by Dowdle et al. (1967) to be a type 1 strain, caused a lesser degree of keratoconjunctivitis and subsequent encephalitis in rabbits than its parent strain, a type 2. Intra-cerebral inoculation of the type 2 caused 89% mortality in mice whereas the type 1 strain produced 30% mortality. Although the comparative data on virulence appears to be credible, the report of selecting

a type 1 strain from a type 2 is puzzling. The parent strain, designated "HF", was originally isolated from recurrent herpes labialis (Flexner and Amoss, 1925) and would be expected, based on its site of isolation, to be type 1. In fact, Dowdle et al., (1967) tested the "HF" strain from two different virus collections (Scott et al., 1957; Ashe and Scherp, 1963) and in each case found the strains to be type 1. Schneeweis (1962) also reported that the "HF" strain in his collection belonged to type 1. Yet, the one tested by Dowdle et al. (1967) from Wheeler's laboratory was undoubtedly type 2. The obvious question at this point is whether Wheeler actually selected one herpes simplex type from another or whether an error was committed in the handling of the strains? Certainly, the capability of either herpes simplex type to convert to the other type is a possibility and must be investigated. Inherent in the necessity for such a study is a need for accurate passage histories to be kept of all strains dealt with and strict measures employed to avoid cross contaminations of strains representing both viral types.

Wildy (1955) reported that two herpesvirus strains which produced small pocks on the egg chorioallantoic membrane were less capable of inducing keratitis or encephalitis than four strains producing large pocks. This report attracted

increased interest when Nahmias (1968) conclusively demonstrated that strains of herpesvirus hominis recovered primarily from genital sites and which belonged to antigenic type 2 were found to produce large pocks (av. dia. 0.5 mm) on the chorioallantoic membrane of embryonated eggs. Type 1 strains, isolated from non-genital sites produced small pocks (av. dia. 0.5 mm). In addition, histological examinations of infected membranes revealed that type 1 strains showed ectodermal proliferation but little subectodermal involvement in their growth pattern. Type 2 strains, however, involved ectodermal, mesodermal, and endodermal layers with marked necrosis and hemorrhages.

Other reports in the literature regarding pock size or histological findings do not take into account repeated egg passages or selection of variants. Coriell (1949) tested primary isolates from throat and skin and found them to produce small pocks and to induce ectodermal proliferation, similar to those described for type 1. Anderson (1940) reported similar findings for a first chorioallantoic generation of a known type 1 strain. After 30 passages in eggs, however, changes similar to those produced by type 2 strains were observed. Using the same strain Dawson (1933) reported that both types of histological lesions were evident after six passages.

The reason for the greater destructive effect of type 2 strains on the chorioallantoic membrane is unclear. One explanation might be the greater ability of type 2 strains to continue growth after two days of incubation. Such growth has been found by Taniguchi (1966) to be interrupted by a presumably type 1 strain isolated from a case of eczema herpeticum.

Plummer and Hackett (1966) found differences in virulence when they compared strains "L2" (type 1) with strain "MS" (type 2) in rabbits and mice. None of 19 adult rabbits infected with the type 1 strain by intramuscular inoculation of the left back leg developed paralysis, while most of the rabbits infected with type 2 became paralyzed. Only a slightly larger percentage of mice showed paralysis from type 2 inoculation than from type 1. In those mice receiving type 2, however, all proceeded to die from encephalomyelitis whereas 38% of those mice paralyzed by the type 1 strain survived although with permanent paralysis. Alford et al. (1967) reported similar finding using freshly isolated type 1 and type 2 strains.

Chzhu-Shan (1959) reported, however, that the type 1 strain "L2" used by Plummer (1966) was more virulent by the intravenous route and less virulent by the intradermal route than a type 2 strain. It would appear from such an observation that pathogenesis of the two types could only be compared when identical

routes of inoculation were employed. Indeed a study of the differences in susceptibility of mice by diverse routes of inoculation and at various ages was made by Johnson (1964a). He used a type 1 strain of herpes simplex virus in his study and so we cannot be certain if the results obtained are also reflective of type 2 pathogenesis. Suckling mice inoculated intracerebrally were highly susceptible to the virus. Encephalitis appeared by the third day post-inoculation and death followed within 24 hrs. The LD₅₀ was determined to be 3 pfu. Fluorescent antibody staining showed antigen only in the central nervous system and, in general, there was no significant extra-neural growth. Infection by intraperitoneal inoculation resulted in a viremia in 2 to 5 hrs. that lasted until the animal died about 5 days later. Virus appeared in the brain 3 days post-inoculation and before it could be detected in the spinal chord. This was in contrast to Cooke et al. (1942) who presented evidence that in rabbits injected intravenously virus spread from infected viscera along afferent nerves to the spinal chord and later reached the brain. Johnson's work strongly indicated a hematogenous spread of virus to the CNS. When mice were inoculated subcutaneously, a few subcutaneous cells and the endoneural cells of subcutaneous nerve fibers were infected. No viremia or visceral infection developed and virus reached the CNS solely by centripetal infection of endoneural cells. After

intranasal inoculation virus gained access to the CNS by multiple neural pathways and by blood-borne infection. These studies strongly indicate that herpes simplex virus can penetrate the CNS by both hematogenous and neural routes. The primary factors determining the pathway were the route of inoculation and the initial site of virus growth, but often more than one pathway was utilized.

In another report Johnson (1964b) suggested that alteration in macrophages of maturing mice plays an important role in development of resistance in older mice. Ten times more virus given intracerebrally was required to kill young adults than newborn mice, whereas, at least 1000 times more virus was needed to kill adult mice by intraperitoneal, subcutaneous, or intra-nasal inoculation.

Interferon production may also play a part in age resistance, since suckling mice appear to be poor producers of interferon (Heineberg, et al. 1964).

A review of the comparative studies available on the virulence of herpes simplex types 1 and 2 indicates that type 2 strains are more virulent. This observation should be confirmed by studying more strains of each type in different animal systems. Experiments on the effect of passage level and selection of variants must also be carried out in a well defined and controlled manner. Whether increased virulence is due to greater

neurotropism, greater growth potentiality, or less susceptibility to host defenses on the part of type 2 strains requires further exploration.

Descriptions of different types of cytopathic effect (CPE) for herpes simplex virus have been reported since the first successful growth of the virus in tissue culture (Scherer and Syverton, 1954). Cray et al. (1958) described three major forms of cytopathic effect on HeLa cells produced by a strain isolated from stomatitis: a proliferative type where cells pile up in a well-defined focus; a non-proliferative type where rounded cells group around an empty space in a cell sheet; and a giant cell type where multinucleated giant cells predominate.

Schneweis (1962) found that type 1 strains in HeLa cells produced foci of small round cells clumped together and appearing as Cray et al. (1958) described as proliferative. Type 2 strains were noted to form aggregates of round cells, usually associated with giant cells. The differentiation of the strains was consistent whether the host cells were HeLa, mouse embryo, chick embryo, or rabbit kidney.

Recently, Ejercito et al. (1968) have classified herpesvirus strains on the basis of cellular changes in Hep-2 cells. Their descriptions of the CPE of the facial and genital strains closely resemble those of Schneweis (1962). Cells infected with the type 1 strain rounded and formed relatively small,

tight, round symmetrical multilayered clumps. The cells forming the clumps were drawn from the immediate vicinity of the initially infected cell. The area surrounding the clump was partially depopulated and in stained preparations appeared as a halo. The type 2 strain caused cells to round and to pile up into spindle-shaped clumps. The clumps tended to fragment with single rounded cells abounding in the vicinity of the clumps.

Cells infected with herpes simplex virus produce intranuclear inclusion bodies. Their appearance and development have been described by Scott et al. (1953) and Love and Wildy (1963). The inclusion body develops in the nucleoplasm by the coalescence of small homogenous masses until the nucleus is filled. The inclusion appears bluish at this time and is Feulgen positive. Later it shrinks from the nuclear membrane, loses its content of nucleoprotein and appears as an eosinophilic mass surrounded by a halo in a nucleus with marginated chromatin. This description was obtained using a type 1 strain. No differences in inclusion body appearance or development of a type 2 strain have been reported.

Investigations of the morphology and development of the herpes simplex particle in infected cells have almost wholly been done with type 1 strains. Wildy et al. (1960) described the virion as having a spherical central area measuring 75 nm in

diameter and containing DNA. A capsid measuring 100 nm in diameter, possessing 5:3:2 axial symmetry and consisting of 162 elongated capsomers surrounds the central area or "core". An envelope derived from the host cell and measuring 145 to 200 nm in diameter surrounds the capsid. Particles emerging from infected cells may or may not possess envelopes and it is uncertain whether or not unenveloped particles are infective. A herpes simplex type 2 strain has also been shown to have this same morphology. Many more unenveloped particles were apparent, however, with the type 2 strain than with a type 1 strain (Figueroa and Hwals, 1968).

Roizman et al. (1963) has reported on herpes simplex infection of cells under one-step growth conditions. Virus complement-fixing antigens appeared within the first two hr accompanied by an increase in the activities of DNA nucleotidyl transferase and deoxyribonuclease. Newly formed viral DNA was detected by the fifth hr. Complete virus particles were evident by the sixth hr, indicating completion of the eclipse phase. Infectivity titers rose rapidly until the ninth hr when they leveled off. No comparative growth studies of type 1 and type 2 strains have yet been reported. Alford et al. (1967) have observed, however, that genital strains of herpes simplex produced one to two logs less infectious virus in primary rabbit kidney, human amnion, HeLa cells, and diploid fibroblasts than did non-genital

strains. If this observation is confirmed, the role of the virus envelope in infectivity may explain some of the difference. Present evidence indicates that there is a close relationship between enveloped particles and infectivity and that non-enveloped particles are incapable or rarely capable of infecting cells (Sprins et al. 1963) Preliminary reports suggest that type 2 strains produce fewer enveloped particles than type 1 strains (Figueroa and Rawls, 1968). A smaller number of enveloped particles could result from a greater instability on the part of type 2 strains. This would be reflected in the inability of a poorly enveloped or unenveloped virion to infect a cell. Type 2 strains have been reported by Schneeweis (1962) to be generally more unstable at 4° C than type 1 strains, although Ejercito et al. (1969) found that facial and genital strains were equally inactivated at 40° C. This latter temperature appears to be artificially high for inactivation studies, however, since it is 3° higher than the temperature of the agent's natural host and 4° higher than its reported optimum growth temperature in tissue culture.

Heparin and other acid mucopolysaccharides appear to affect the initial attachment of herpes simplex type 1 strains to mammalian cells (Mahmias and Kibrick, 1964; Vaheri, and Cantell, 1963; Takemoto and Fabisch, 1964). More interesting, from a comparative viewpoint, is the report of Lehel and Madhazy (1966)

that heparin inhibited the production of skin lesions in rabbits by five herpes strains isolated from cases of herpes labialis, while a strain from a recurrent thigh lesion was more resistant to heparin. Such variation in susceptibility to heparin could be indicative of differences between type 1 and type 2 strains at the envelope level even though data on quantitative differences in the envelopes is unavailable.

Russell and Crawford (1964) have isolated, purified and analysed the DNA from a type 1 strain. They have shown that it is double stranded, has a density of 1.727 g/ml and a molecular weight of 6.8×10^6 . The proportion of guanine + cytosine to adenine + thymine was 68%. The G + C ratio of a type 1 strain analyzed by Ben-Porat and Kaplan, (1962), however, was reported to be 72%. One laboratory has reported that the buoyant densities of four type 1 strains ranged from 1.253 to 1.271 g/cm³ whereas a buoyant density of 1.266 - 1.267 g/cm³ was reported for each of three separate type 2 strains (Schiek, 1967). In all, the various antigenic and biologic differences related thus far suggest that the genomes of the two types differ. Comparative studies on molecular weight buoyant density, G + C ratio, and DNA homology of numerous strains of each type should be undertaken to determine if, indeed, a genetic difference exists.

There is still much to be learned about differences between

the two herpes simplex types at the serological, biological and physical level. It must be emphasized that findings with one strain do not necessarily reflect a universal characteristic of the relative serotype. The effect of virus passage may enhance selection or mutation and affect certain characteristics. This has already been made apparent and was mentioned previously in regard to animal virulence studies, growth on the chorioallantoic membrane, and in selection of variants. The frequency with which variants appear and the degree of difference they possess from their parent strains may even add to the knowledge of the evolutionary development of herpes simplex virus. All studies of the relative role of the two herpes simplex types in human disease, perhaps even cancer (Hawls et al., 1968), will shed light on all the medical problems associated with this agent.

MATERIALS AND METHODS

Growth Media for Tissue Cultures

Medium 199 containing almost all of the amino acids and vitamins as well as a number of nucleic acid constituents, growth factors and intermediary metabolites in a Hanks balanced salt solution base was purchased from Grand Island Biological Co. Grand Island, N. Y. and used exclusively in this work. Added to this commercial preparation were 100 mg/L glutamine (Cibco, Grand Island, N. Y.), 250,000 units/L buffered potassium penicillin (Lilly, Indianapolis, Ind.), and 250 mg/L streptomycin sulfate (Lilly, Indianapolis, Ind.). Initiator medium, used for preparation of primary tissue culture, contained 1% sodium bicarbonate and 10% lamb serum (Microbiological Associates, Bethesda, Md.) in addition to the supplemented Medium 199 just described. Maintenance medium contained Medium 199, 2.5% sodium bicarbonate and 5% lamb serum and was used for maintaining tissue culture after it was established as a monolayer. The diluent used in all experiments contained Medium 199, 1% NaHCO_3 , and no serum. Overlay medium for plaque assay contained 2.5% methylcellulose (1500 centipoise) in maintenance medium. Methylcellulose was purchased from Fisher Scientific, Fair Lawn, N.J. It produces a semi-solid gel which permits plaques to appear in the cell monolayer rather than a disseminated cyto-

pathic effect.

Tissue Cultures

Primary rabbit kidney tissue culture was prepared by aseptically removing the kidneys of a rabbit less than 3 weeks old, mincing them well with sharp scissors and trypsinizing in a bevel sided flask to aid disruption. Thirty ml of a 2.5% solution of pancreatic trypsin digest (1:250) obtained from Difco, Detroit, Mich. and dissolved in saline was used per pair of kidneys. The tissue suspension was spun at a speed just below the level at which foaming occurs on a magnetic stirrer for 30 min, pelleted by centrifugation at 300g for 10 min, and resuspended in 400 ml of 199 initiator medium. The suspension was distributed as desired, either 2 ml in tissue culture tubes (Kimex, 15 ml), 5 ml in tissue culture plates (60X15mm, Falcon plastics), or 30 ml in 16 oz prescription bottles. Tubes and bottles were tightly stoppered (white rubber, Aloe) and incubated at 37° C. Plates were held at the same temperature but in a moist, 5% CO₂ atmosphere.

Primary mouse embryo tissue culture was prepared in the same manner with three exceptions; initiator medium contained 15% serum; the trypsinized tissue from the litter of one pregnant mouse was suspended in 150 ml; and 0.25% trypsin was used rather than 2.5%. Human embryo fibroblasts were obtained from Microbiological Associates, Bethesda, Md.

Viruses

The virus strains used in this study and their site of isolation are presented in Table 1.

Stocks of individual virus strains were prepared by inoculating the cell monolayer contained in a 16 oz prescription bottle with a high multiplicity of virus. Infection was allowed to proceed until approximately 60% of the cells showed a cytopathic effect. The bottle was frozen and thawed once and the contents clarified by low speed centrifugation. The supernatant liquid containing most of the virus, was dispensed in 0.5 ml volumes in 1.0 ml glass ampules (Kimex, Scientific Products) which were heat sealed and store at -70° C.

Virus Assay

Viral suspensions were titered by the plaque assay technique. Serial 10-fold dilutions in 199 maintenance medium were made and from each dilution, 0.2 ml was absorbed onto a tissue culture plate for 15 min, shaking once after ten min had elapsed. Two plates were used per dilution. Unless stated otherwise, all viral assays were done using rabbit kidney tissue culture. At the end of the adsorption period the plates were overlaid with methylcellulose medium and incubated in a 5% CO₂ atmosphere at 37° C. After 48 hr incubation plaques were counted microscopically on an inverted microscope. The plate on which plaques were to be counted was placed on a lined petri dish to facilitate

counting. Viral titers are expressed as plaque forming units (pfu) per 0.2 ml.

Antisera

Antisera were prepared against strains L2 and MS representing herpes simplex types 1 and 2, respectively. Three month old, white, New Zealand rabbits were inoculated intramuscularly in the left rear leg and intraperitoneally with 0.5 ml volumes of heavily infected tissue culture fluid. Three weeks later the same injection procedure was repeated. One week after that the animal was bled by means of an ear bell attached to a vacuum pump. The whole blood was allowed to clot overnight at 4° C and the serum was separated by centrifugation the following day. Antiserum was dispensed in 0.5 ml amounts and stored at -20° C in sealed glass ampules.

Human antiserum was obtained by bleeding an individual suffering from recurrent cold sores one week after an eruption had occurred.

Neutralization Tests

Two types of neutralization tests were employed in this study. One was the standard doubling dilution test by which the ability of an antiserum to neutralize a given amount of virus is measured. In this technique, 0.5 ml of serum was serially diluted in two fold dilutions using 199 diluent; 0.5 ml of virus suspension containing 200 pfu/0.2 ml was then added to each

dilution tube so that the final virus concentration in each tube was 100 pfu/0.2 ml. A control tube included in each test consisted of 0.5 ml virus suspension and 0.5 ml 199 diluent containing 1% pre-inoculation rabbit serum. All tubes were incubated for 1 hr in a 37° C water bath with periodic shakings every 15 min. At the end of the incubation period, 0.2 ml was withdrawn from each tube and assayed for virus using rabbit kidney tissue culture plates. The titer of the serum was judged to be the highest dilution which completely neutralized all of the virus.

Neutralization curves of the type described by Plummer (1964) were used to test for serological differences between strains of herpes simplex, types 1 and 2. All experiments were performed using 199 diluent. One ml of virus suspension containing approximately 2000 pfu was mixed with 1 ml of antiserum. The mixture was kept in a 37° C water bath and assayed for virus at 5 min, 20 min, and 40 min. A control tube, consisting of 1 ml of the same virus suspension mixed with 1 ml of 199 diluent, was incubated with the test preparation. The control was assayed at the start of the incubation period and after 40 min of incubation.

Growth Curves

Each of several tissue culture tubes was inoculated with 0.2 ml medium containing 500 pfu of virus. The inoculum was adsorbed for one hour at 37° C. At the end of the adsorption

period each tube was washed 2 times with 199 diluent to remove unadsorbed virus, overlaid with 1 ml of 199 maintenance medium and the incubation continued. At hourly intervals the supernatant fluids from two tubes were pooled and assayed for infectious virus. The cell sheets were washed 2 times with 199 diluent, scraped off with a rubber policeman, pooled into a 1ml suspension in 199 maintenance medium and disrupted in a Ten-Broeck grinder (Aloe) to release cell associated virus. This suspension was clarified by centrifugation at 300g for 10 min and the supernatant fluid assayed for infectious virus.

In order to accurately measure the inoculum two tissue culture plates were inoculated and adsorbed in the same manner and at the same time as the tubes. After the 1 hr adsorption period the plates were overlaid with methylcellulose medium to induce plaque formation. The number of plaques counted two days later was considered to be the inoculum per tube.

Inactivation Curves

Cell monolayers in 16 oz prescription bottles were infected with virus. When 50% of the cell sheet showed a cytopathic involvement, the supernatant fluid was poured off and the cells were washed 3 times with 199 diluent. The cell sheet was then scraped off using a rubber policeman and suspended in 3 ml of 199 diluent where it was disrupted in a Ten-Broeck grinder. The

disrupted material was passed consecutively through two separate Millipore filters, 3.0u and 0.45u and 13 mm in dia. and diluted 1:10 in 199 diluent. This preparation was then enclosed in a tightly stoppered 4 oz bottle and held in a 37° C water bath. The supernatant fluid from the infected bottle was filtered and diluted in exactly the same manner. Periodically samples were removed from each bottle and assayed for the presence of infectious virus.

Neurovirulence studies

Two separate experiments were designed to investigate the comparative neurovirulent properties of herpes simplex, types 1 and 2.

In the initial experiment, viral strains representative of herpes simplex types 1 and 2 were used to inoculate 5 month old New Zealand white rabbits and 3 week old white Swiss mice. The femoral muscle of the left back leg of both rabbits and mice was injected with 10^4 pfu and 10^3 pfu respectively. The animals were observed for the occurrence of paralysis of myelitis and, when possible, histologic preparations of pathologically affected tissues were made of the spinal cord and dorsal ganglion. Sections were cut on a cryostat and stained with hematoxylin and eosin.

The other experiment consisted of performing parallel

titrations in 3 different tissue culture systems and in 3 week old white Swiss mice. Virus grown in rabbit kidney cell culture was simultaneously assayed in rabbit kidney, human fibroblast, and mouse embryo tissue culture as described previously. Ten mice were inoculated per dilution intracerebrally by injection of 0.05 ml of virus suspension. The number of dead animals after ten days observation was used in the calculation of the LD₅₀ by the Kaerber Method:

$$LD_{50} = 0.5 + \log \text{ of highest concentration of virus used } - \frac{\text{sum of per cent of dead animals}}{100}$$

DNA extraction

DNA was extracted from infected cells by a modification of the method of Marmur (1961). Tissue culture monolayers in 16 oz prescription bottles were inoculated with high multiplicities of virus. When 60% of the cells showed a cytopathic involvement, the cells were scraped off with a rubber policeman, pooled, and pelleted by centrifugation at 500g. A minimum of 0.1 ml packed cells was required for the extraction treatment. The packed cells were resuspended in 2.5 ml EDTA buffer (0.1M and PH8.0) and treated as follows:

1. Added to the cell suspension were 0.6 ml of a 10% sodium dodecyl sulfate solution. The suspension was gently shaken for 1-3 min.

2. Added next was 0.6 ml 5M NaClO₄. The solution was shaken for 1-3 minutes.

3. At this point, the suspension was deproteinized by the addition of 2.5 ml of chloroform-isoamyl alcohol (24:1 V/V

4. The resulting emulsion was separated into three phases by centrifugation at 1500g for 15 min. The top aqueous phase contained DNA. The middle interphase contained protein and the bottom phase contained the chloroform isoamyl alcohol mixture,

5. The aqueous phase was removed with a widebore pipette and the bottom phases were discarded.

6. 2-4 volumes of absolute ethyl alcohol were added to the separated aqueous phase and mixed.

7. At this point, DNA was lifted out by spooling it on to hooked glass rod. It was dissolved in 2.5 ml of ml of 0.10 saline citrate (SSC). SSC consisted of a 0.15 NaCl solution containing 0.15M trisodium citrate. The PH was 7.0.

8. Steps 3-7 were repeated. An indication of the concentration and purity of the preparation was obtained by measuring the optical density of the final DNA preparation at 230, 260, and 280 mm.

DNA preparations could be stored conveniently at 40° C for short periods of time and at -20° C indefinitely.

Procedure for Density Gradient Analysis

The base compositions of 3 strains of herpes simplex

type 1 and 3 strains of type 2 were determined by measurement of buoyant density in cesium chloride density gradients. The viral DNA densities were calculated from the positions of the U.V. absorbing bands relative to marker DNA.

Analysis of extracted viral DNA for density was performed using a Beckman Spinco Model E centrifuge equipped with monochromator for ultraviolet absorption studies and a photo-electric scanner. All centrifugations were made at 25° C, 44,000 rpm, using the AM-D rotor and double-sector 2° aluminum-filled Epon centerpiece. CsCl of optical quality was used.

Density gradient bands on the tracings were analyzed on a DuPont Curve resolver. This is an analog computer that finds the component Gaussian curves in a complex curve. Density at the mid-point of a Gaussian DNA band is found by measuring the distance from an appropriate density marker, using the slope of the gradient from calibration curves done with known bacterial DNA.

Base composition (G+C%) was calculated from density making use of the linear relation between density and base composition (R).

$$G+C(\%) = \frac{\text{density} - 1.660}{0.098} \times 100$$

CHAPTER III

Results-Serological Studies

Antibody Formation in Infected Rabbits

The rate of antibody formation in rabbits injected with $3.3 \log_{10}$ pfu of type 1 and type 2 herpes simplex viruses is presented in Fig. 4. The titers of the sera specimens were determined by the doubling dilution neutralization test and are plotted against the days on which the samples was taken. The curve presented is typical of those obtained from immunization experiments done using three different strains of type 1 and three of type 2 viruses. The first detectable neutralizing antibody appeared at 12 days post-inoculation. There was a gradual increase through 21 days when titers reached a level of approximately $1/16$ - $1/32$. At three weeks a secondary response was elicited by repeating the original inoculation procedure. In one week antibody titers had increased to levels between $1/256$ and $1/512$. It was at this time that the animals were bled and the serum used for identification purposes.

Identification of Herpes Simplex Virus Strains

All of the virus strains listed in Table 1 were identified as to type by neutralization curves using antiserum prepared in rabbits and virus grown in rabbit kidney cells. Strain "L2" was isolated by Shubladze (1960) and strain "MS" by Gudnadottir

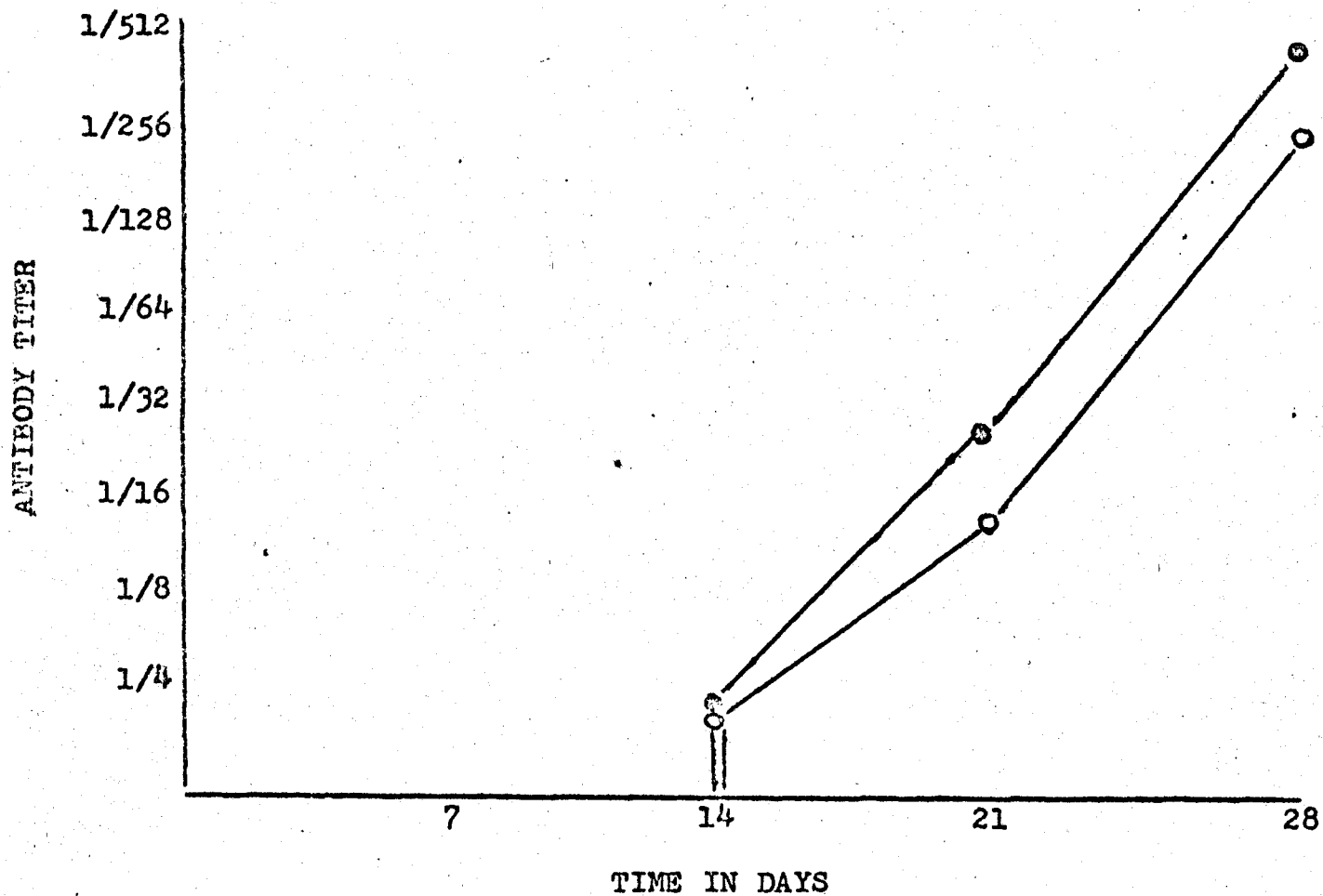


Fig. 4 Antibody production in rabbits of herpes simplex types 1 and 2.
○—○ = Type 1 antibody
○—○ = Type 2 antibody

Table 1

Virus strains and their sites of isolation

<u>Type 1</u>		<u>Type 2</u>	
<u>Strain</u>	<u>Origin</u>	<u>Strain</u>	<u>Origin</u>
L2	Lip	MS	CNS
197	Mouth	Wiggins	Genital
Watson	Lip	Dawson	Genital
356	Pharynx	Willis	Genital
Glover	Eye	D64	Generalized infection of an infant
E515	Mouth	ST-1	Genital
Slatton	Brain	ST-2	Genital
Crowe	Face	ST-3	Genital
Bramson	Mouth	Howard	Genital
Hines 100	Mouth	Jansen	Genital
		Sandra	Genital
		Johnson	Genital
		Fugi	Genital

(1964). Both of these strains have high, but unknown passage histories. Strains 197, Watson, 356, Clover, E515, Slatton, Wiggins, Dawson, Willis and D64 were obtained from the Communicable Disease Center and had been passed no more than four, and no less than two, times in rabbit kidney tissue culture. Strains Crowe, Bramson, Hines 100, ST-1, ST-2, ST-3, Howard, Jansen, Sandra, Johnson and Fugi were isolated in our laboratory and were tested for type specificity after one passage in rabbit kidney culture.

Typical neutralization curves of both herpes simplex serotypes grown in rabbit kidney cells and neutralized by rabbit type 1 and type 2 antiserum appear in Figs. 1A and 2A respectively. The slope of the curve for the homologous virus is much steeper than that for the heterologous virus indicating more rapid neutralization. Neutralization of all homologous virus was completed by 20 min, whereas, heterologous virus was still detectable at 40 min. Our determinations of the serologic type specificity of the strains listed in Table 1 correlated perfectly with their site of isolation. This confirmed the original report of Dowdle et al. (1967), that type 2 strains are isolated primarily from the genitalia whereas type 1 strains are recovered from other areas of the body.

Three type 1 and three type 2 strains were carried through 30 consecutive passages in rabbit kidney cells to determine if

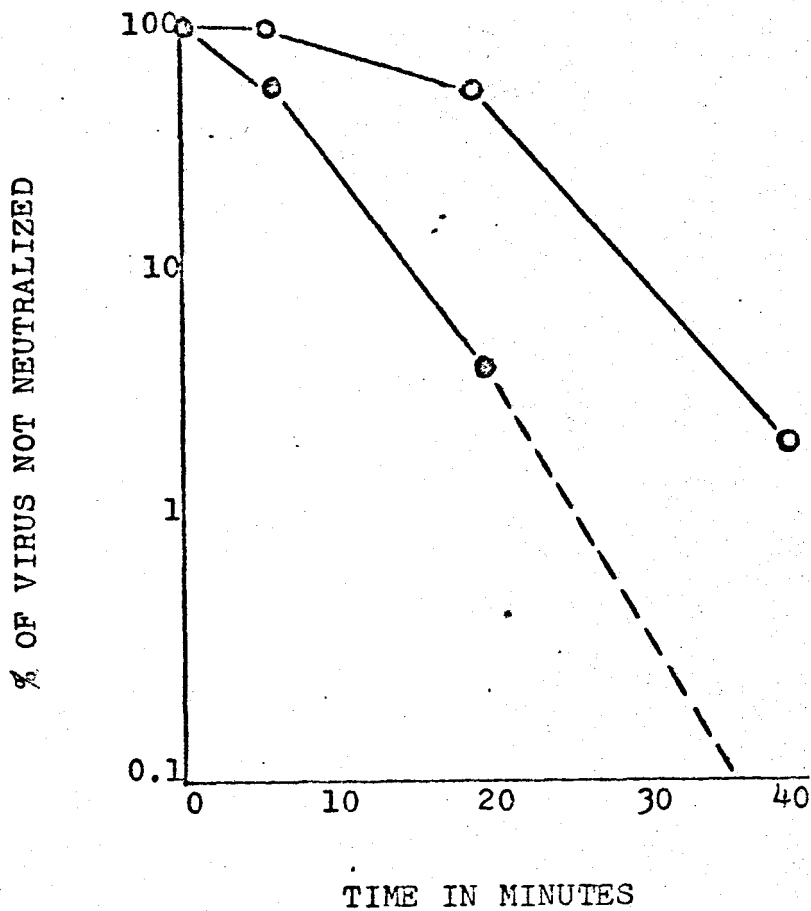


Fig. 1A Neutralization of virus grown in rabbit kidney cells by rabbit antiserum prepared against type 1 virus.

●—● = Type 1 virus

○—○ = Type 2 virus

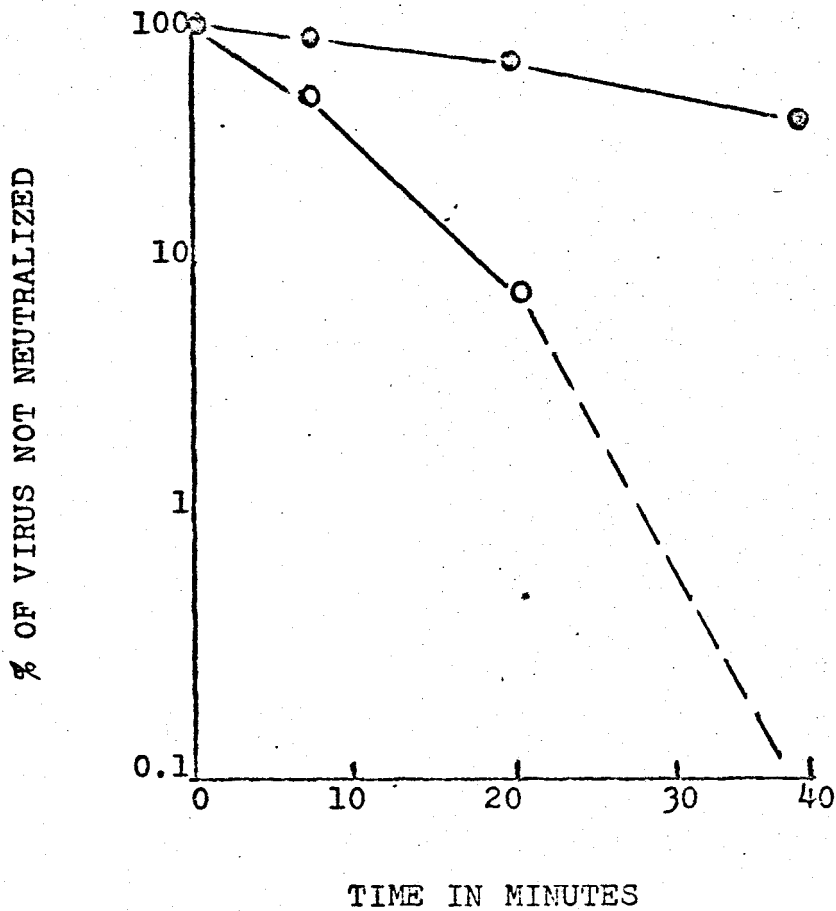


Fig. 2A Neutralization of virus grown in rabbit kidney cells by rabbit antiserum prepared against type 2 virus.

●—● = Type 1 virus

○—○ = Type 2 virus

the serologic specificity of the two herpes simplex types was a stable characteristic, and no conversion from one serotype to the other occurred. Neutralization curves done after passage were identical to those in Figs. 1A and 2A indicating no change in serological properties.

Serological Properties of Herpes Simplex Type 1 and Type 2 Grown in Tissue Other Than Rabbit Kidney.

Herpes simplex types 1 and 2 could be easily distinguished serologically with rabbit type 1 antiserum when they were grown in mouse or human fibroblasts (Figs. 1B, 1C). Rabbit type 2 antiserum was effective in identifying virus grown in mouse fibroblasts but was less effective on virus grown in human fibroblasts (Figs. 2B, 2C). Type 1 and type 2 viruses grown in rabbit or mouse brain could be identified using type 1 antiserum (Figs. 1D, 1E), but were indistinguishable with type 2 antiserum (Figs. 2D, 2E). To insure that brain constituents were not interfering with neutralization, neutralization curves were done on type 1 and type 2 virus grown in rabbit kidney cells and mixed with a homogenate of uninfected rabbit brain. The results of this experiment in Figs. 1F and 2F show a typical neutralization curve for rabbit kidney grown virus indicating no inhibitory effect by brain constituents.

In view of the results obtained using virus grown in rabbit and mouse brain, neutralization curves were done on

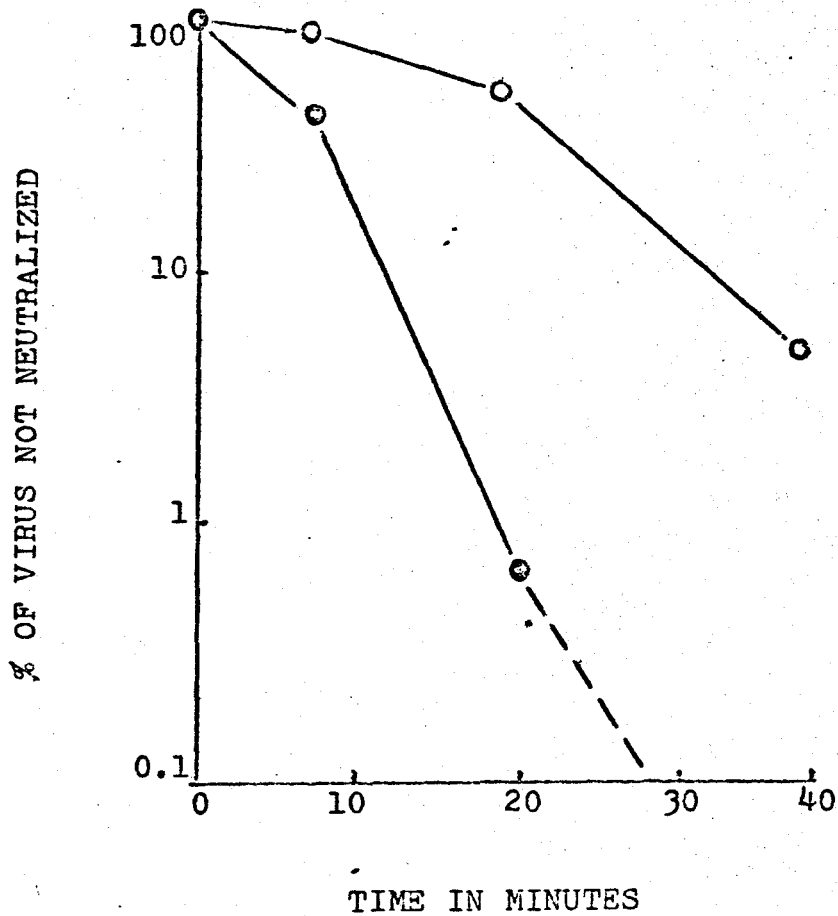


Fig. 1B Neutralization of virus grown in mouse fibroblasts by rabbit antiserum prepared against type 1 virus.

●—● = Type 1 virus

○—○ = Type 2 virus

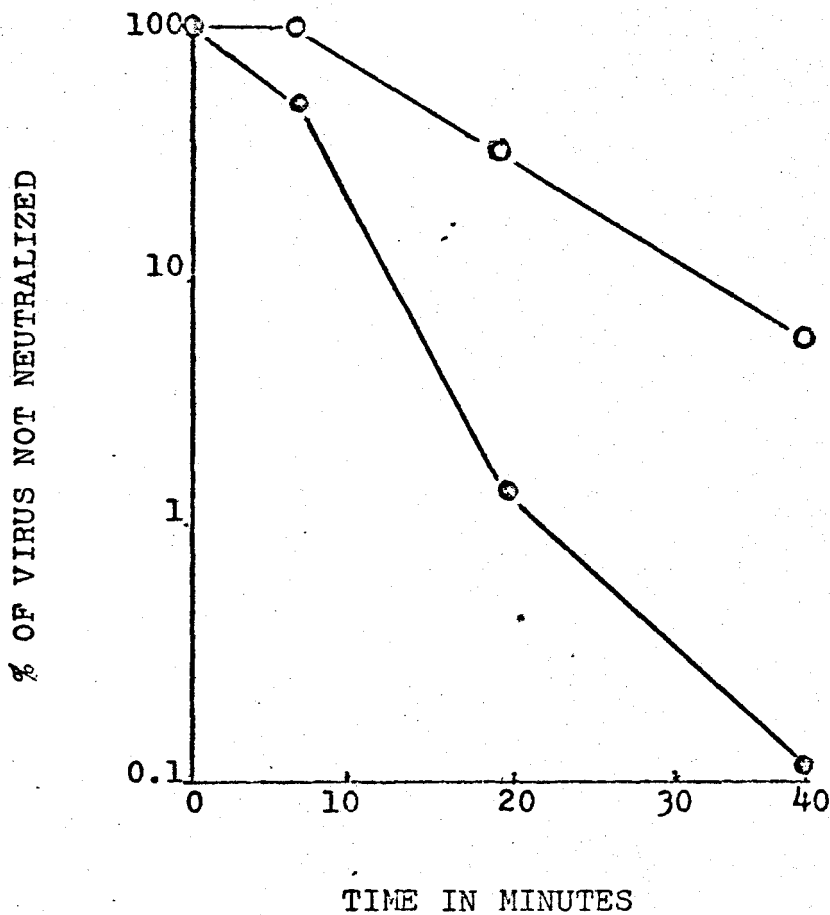


Fig. 1C Neutralization of virus grown in human fibroblasts by rabbit antiserum prepared against type 1 virus.

●—● = Type 1 virus

○—○ = Type 2 virus

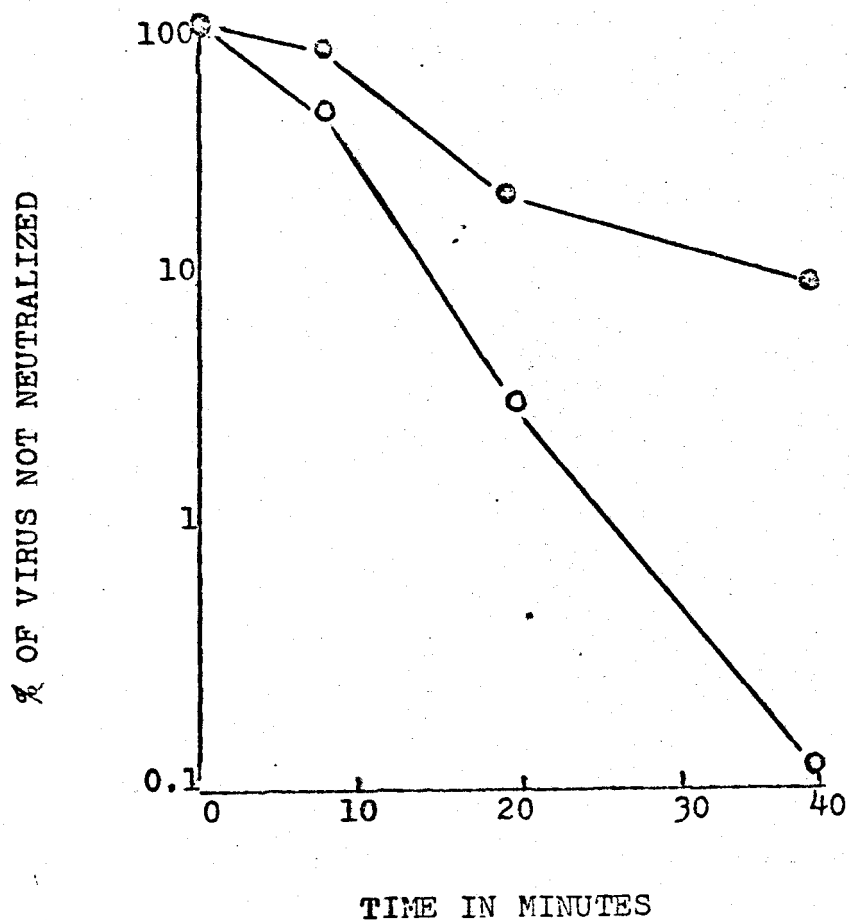


Fig. 2B Neutralization of virus grown in mouse fibroblasts by rabbit antiserum prepared against type 2 virus.

●—● = Type 1 virus

○—○ = Type 2 virus

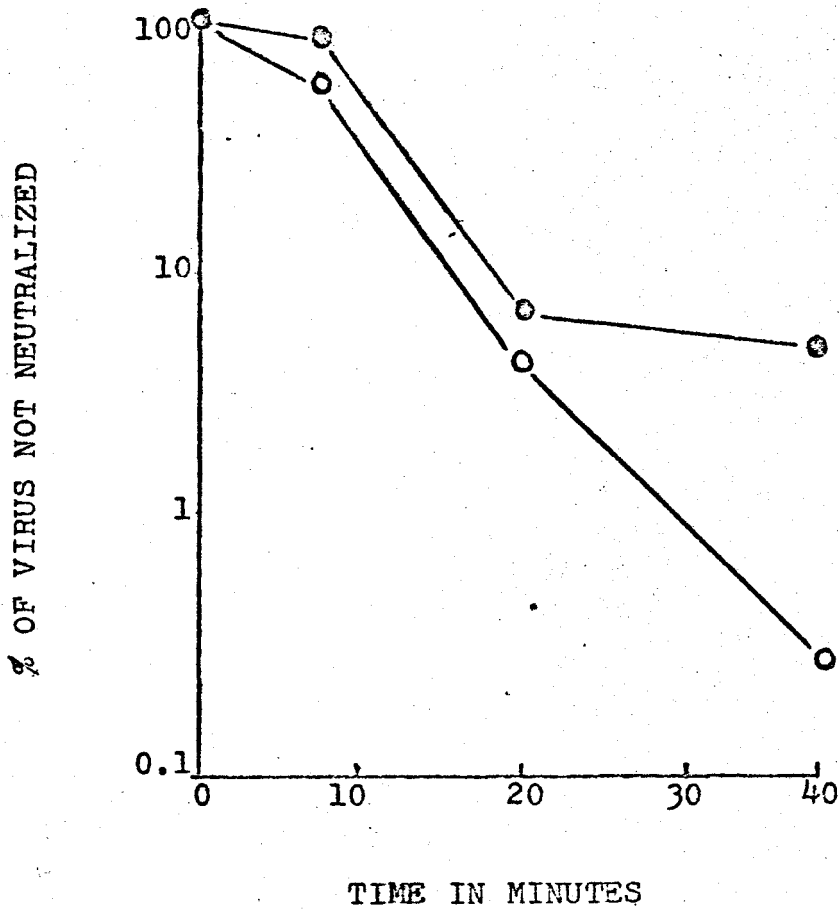


Fig. 2C Neutralization of virus grown in human fibroblasts by rabbit antiserum prepared against type 2 virus.

●—● = Type 1 virus

○—○ = Type 2 virus

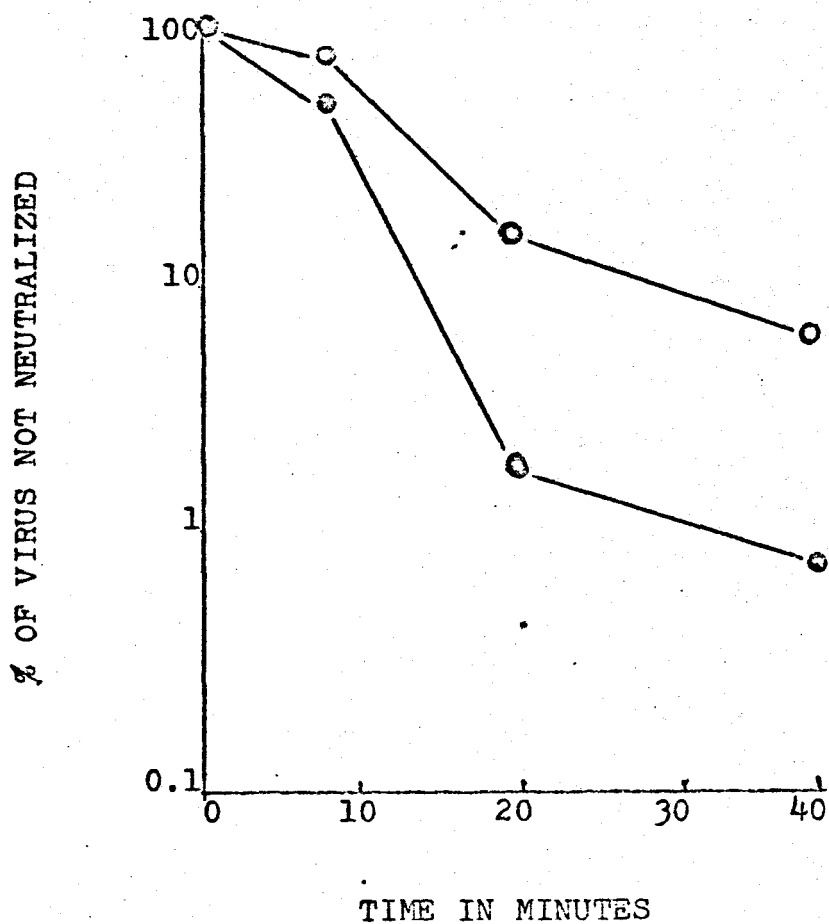


Fig. 1D Neutralization of virus grown in rabbit brain by rabbit antiserum prepared against type 1 virus.

●—● = Type 1 virus

○—○ = Type 2 virus

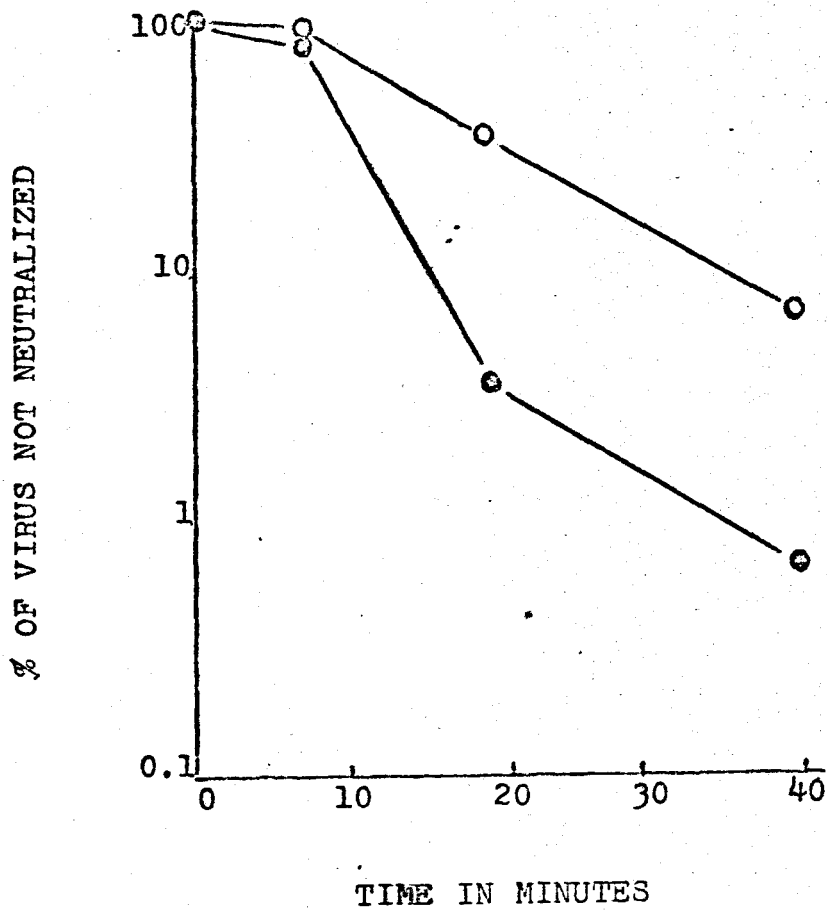


Fig. 1E. Neutralization of virus grown in mouse brain by rabbit antiserum prepared against type 1 virus.

○—○ = Type 1 virus

●—● = Type 2 virus

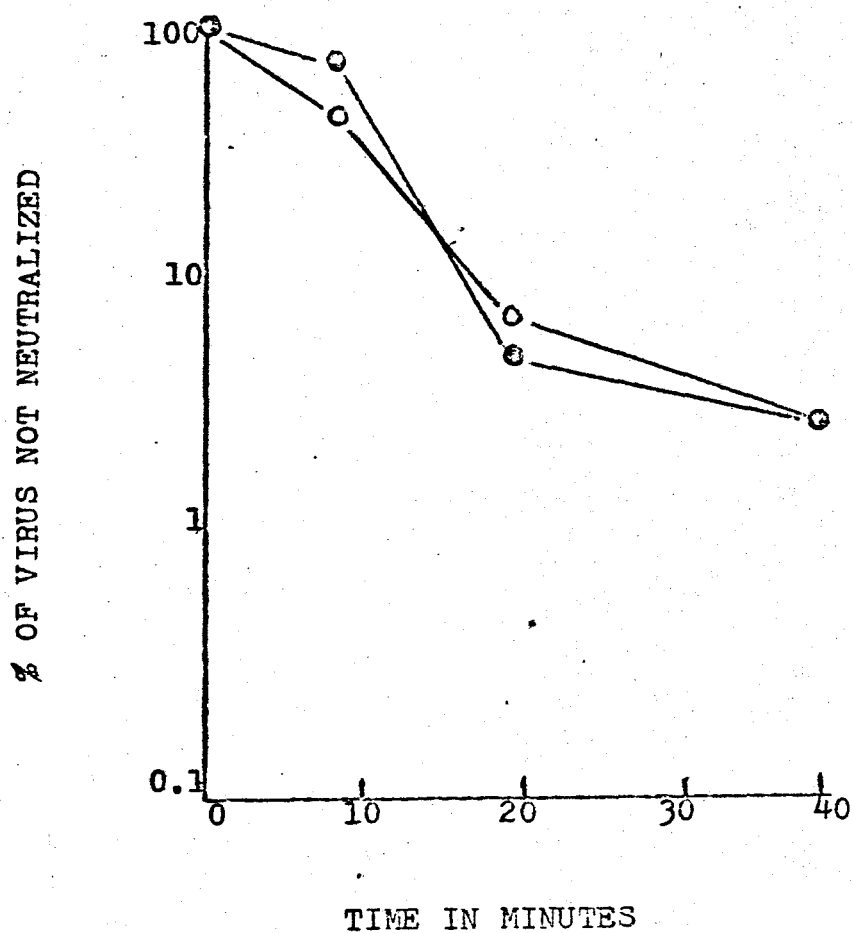


Fig. 2D Neutralization of virus grown in rabbit brain by rabbit antiserum prepared against type 2 virus.

○—○ = Type 1 virus

●—● = Type 2 virus

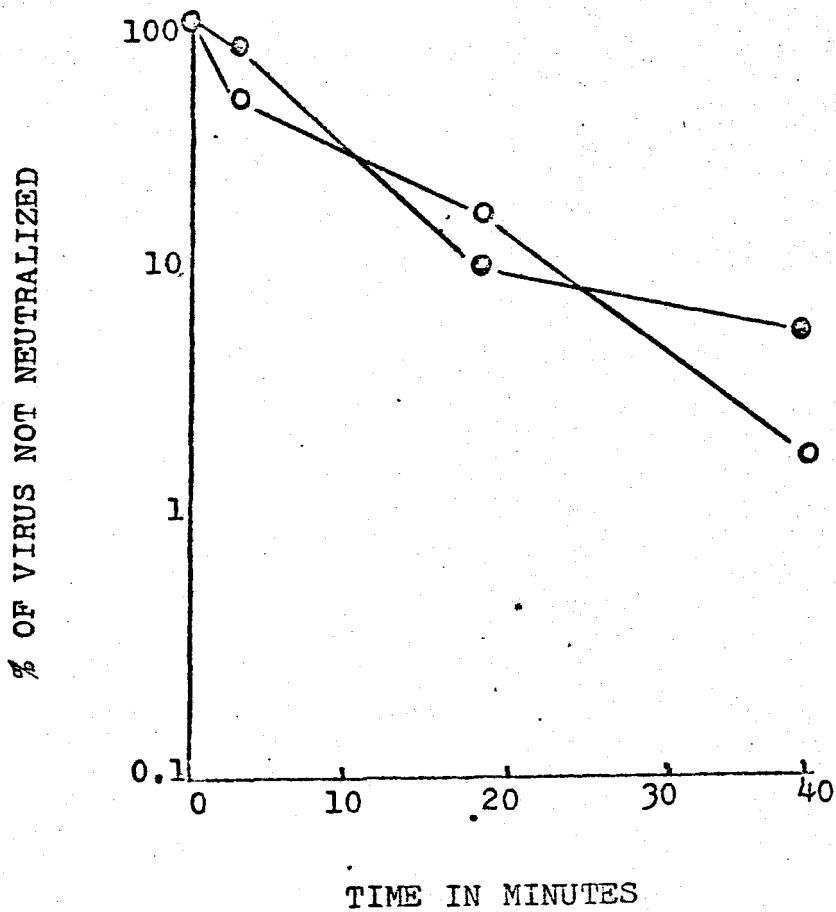


Fig. 2E Neutralization of virus grown in mouse brain by rabbit antiserum prepared against type 2 virus.

●—● = Type 1 virus

○—○ = Type 2 virus

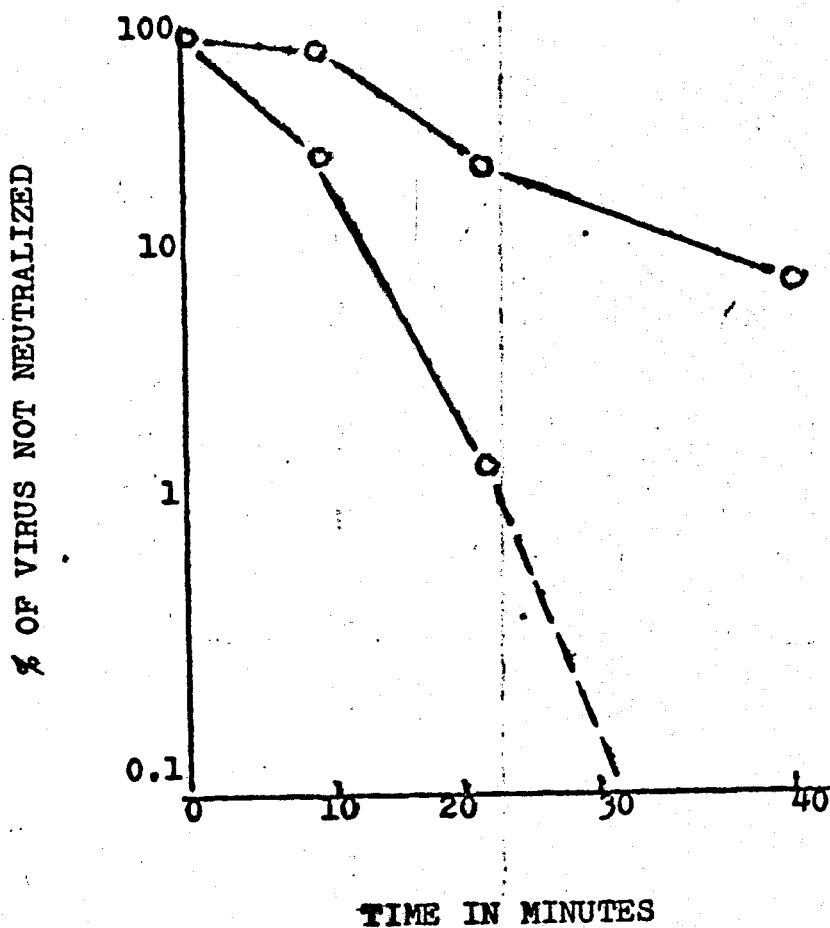


Fig. 1F Neutralization by rabbit antiserum prepared against type 1 virus of virus grown in rabbit kidney cells and mixed with rabbit brain

●—● = Type 1 virus

○—○ = Type 2 virus

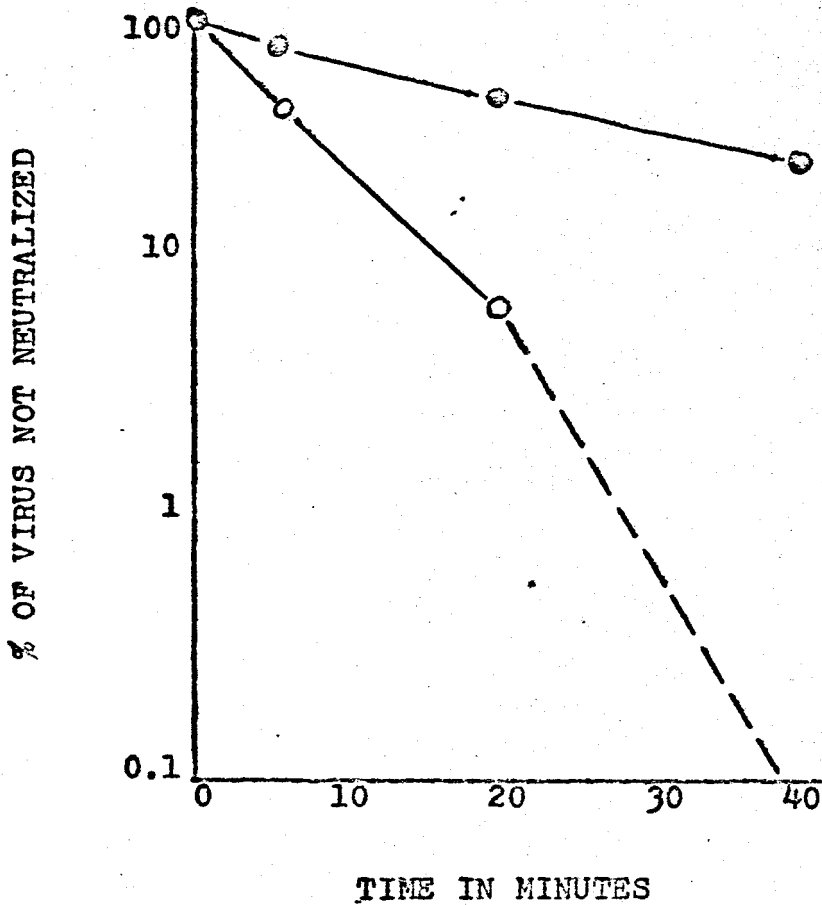


Fig. 2F Neutralization by rabbit antiserum prepared against type 2 virus of virus grown in rabbit kidney cells and mixed with rabbit brain

0—0 = Type 1 virus

0—0 = Type 2 virus

this virus after it had been grown again in rabbit kidney cells. The resulting curves were identical to those in Figs. 1A and 2A of rabbit kidney grown virus indicating no selection of a variant during growth in the animal brains.

Antiserum used in neutralization curves from an individual with recurrent cold sores could readily distinguish virus grown in rabbit kidney cells, mouse fibroblasts and human fibroblast (Figs. 3A, 3B, 3C). It could not however, identify as easily virus grown in rabbit or mouse brain (Figs. 3D, 3E).

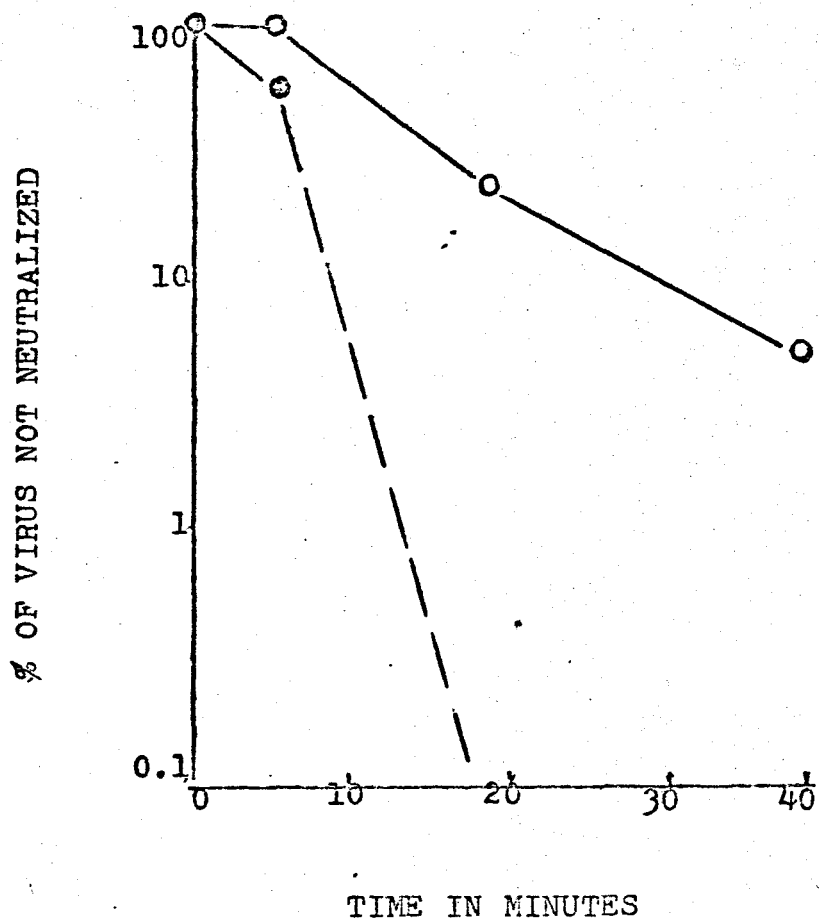


Fig. 3A Neutralization of virus grown in rabbit kidney cells by human type 1 antiserum.

---○ = Type 1 virus

—○ = Type 2 virus

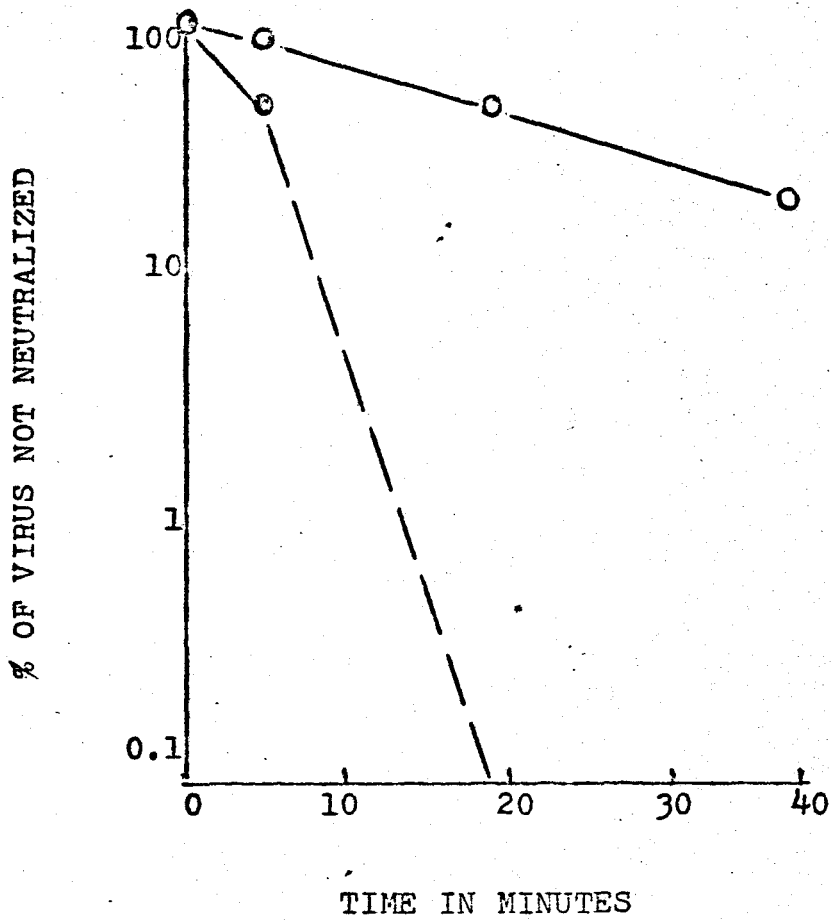


Fig 3B Neutralization of virus grown in mouse fibroblasts by human type 1 antiserum.

○—○ = Type 1 virus

○—○ = Type 2 virus

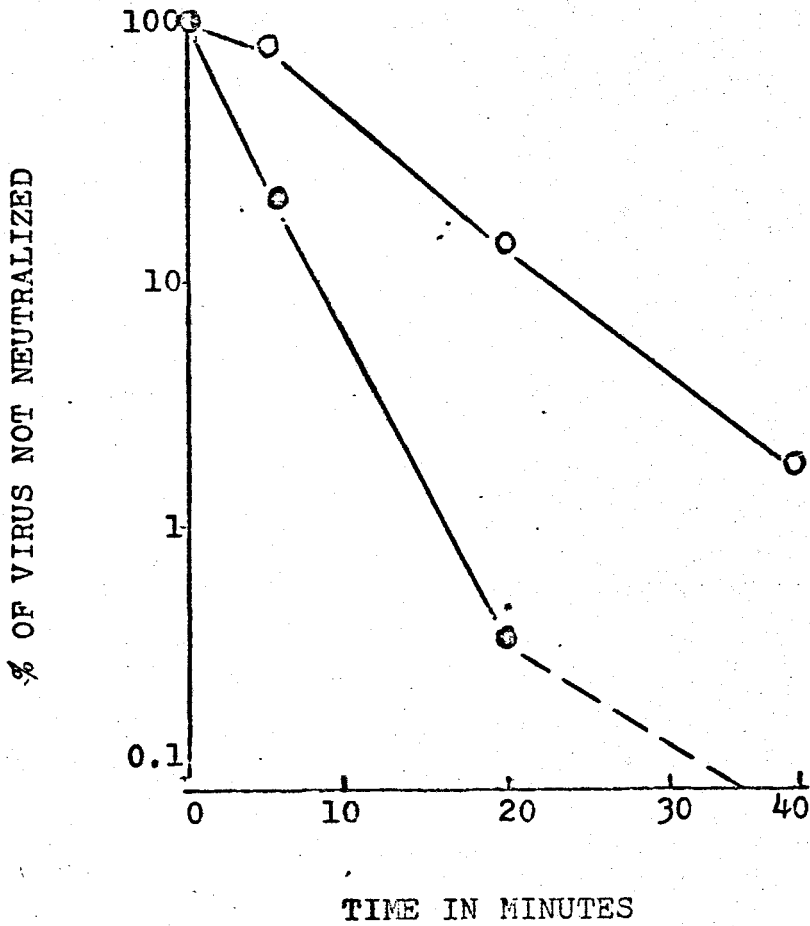


Fig. 3C Neutralization of virus grown in human fibroblasts by human type 1 antiserum.

●—● = Type 1 virus

○—○ = Type 2 virus

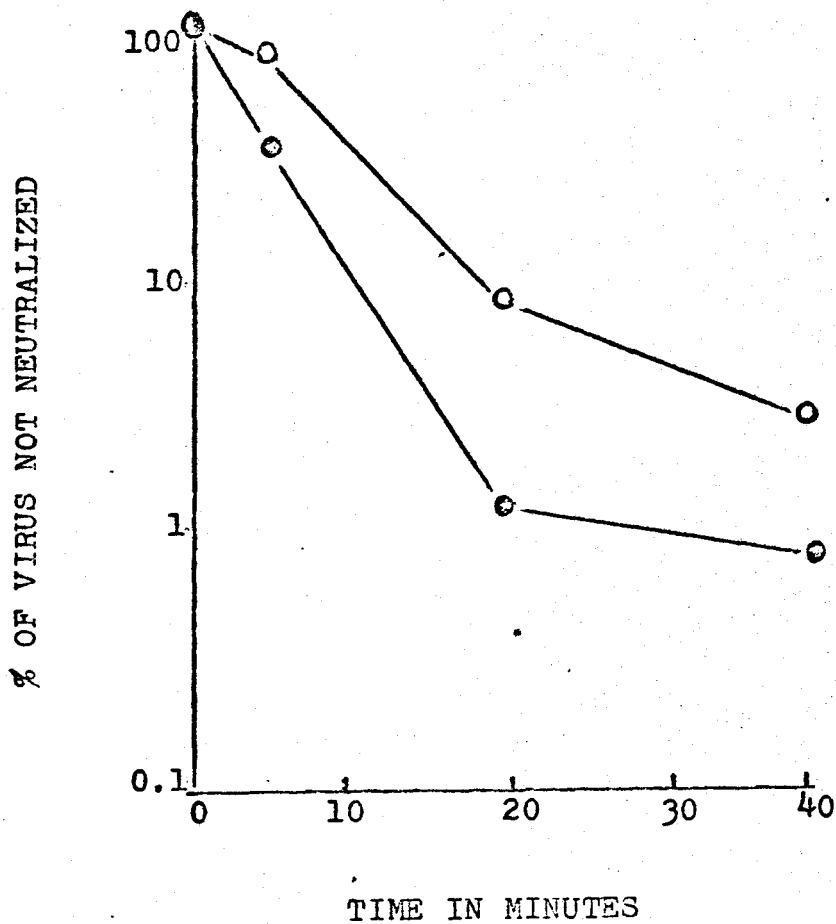


Fig 3D Neutralization of virus grown in rabbit brain by human type 1 antiserum.

●—● = Type 1 virus

○—○ = Type 2 virus

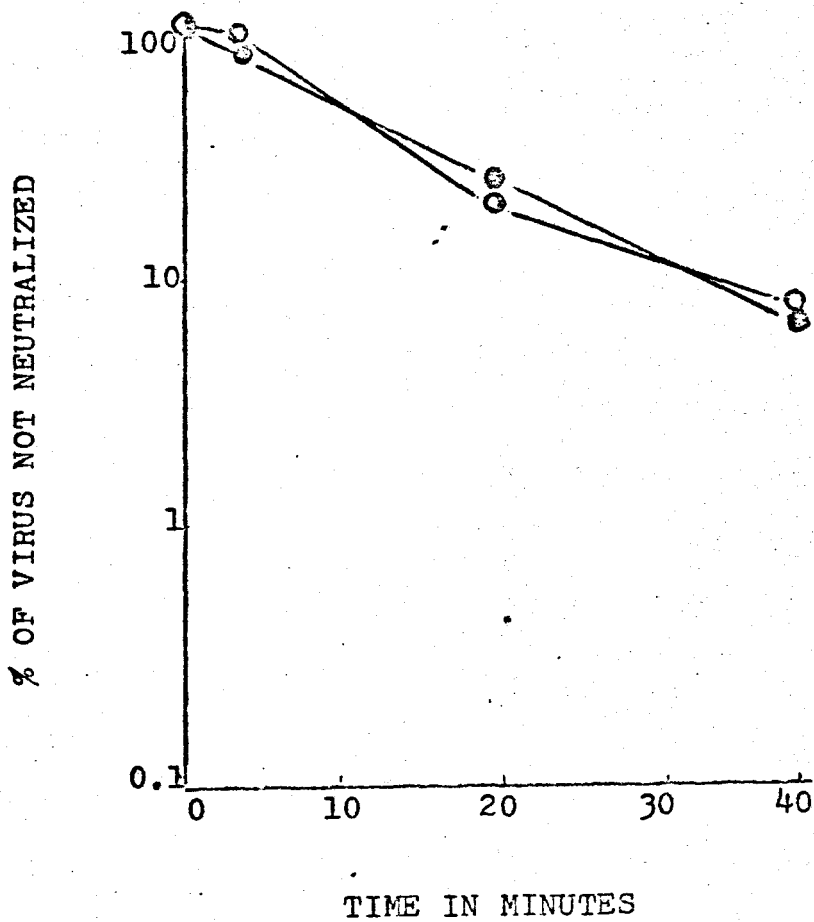


Fig. 3E Neutralization of virus grown in mouse brain by human type 1 antiserum.

●—● = Type 1 virus

○—○ = Type 2 virus

CHAPTER IV

Results-Physical Studies

Stability at 37° C

Type 2 virus grown in either rabbit kidney cells or in mouse fibroblasts was more readily inactivated at 37° C than was type 1 virus. The greater instability of type 2 was apparent whether cell associated virus or virus from culture fluid was assayed (Figs. 5A,B,C,D). Herpes simplex types 1 and 2 were more alike in their rates of inactivation when they were grown in human fibroblasts, although type 2 virus was still more unstable (Figs. 5E, and F).

Herpes virus particles are released slowly from infected cells and may acquire their envelopes from different cellular membrane structures (Darlington and Noss, 1968). In order to investigate whether virus released early in the growth cycle differed in stability from particles released later, rabbit kidney tissue cultures infected with type 1 and type 2 viruses were washed 3 times with 199 diluent at 22 hr, 46 hr, and 70 hr post inoculation and overlaid with fresh maintenance medium. Four hr later the medium was removed. The virus released into the tissue culture fluid during the four hr period was titered immediately and divided into three parts. One part was placed at -70 C, one at 4 C, and one in 37 C water bath.

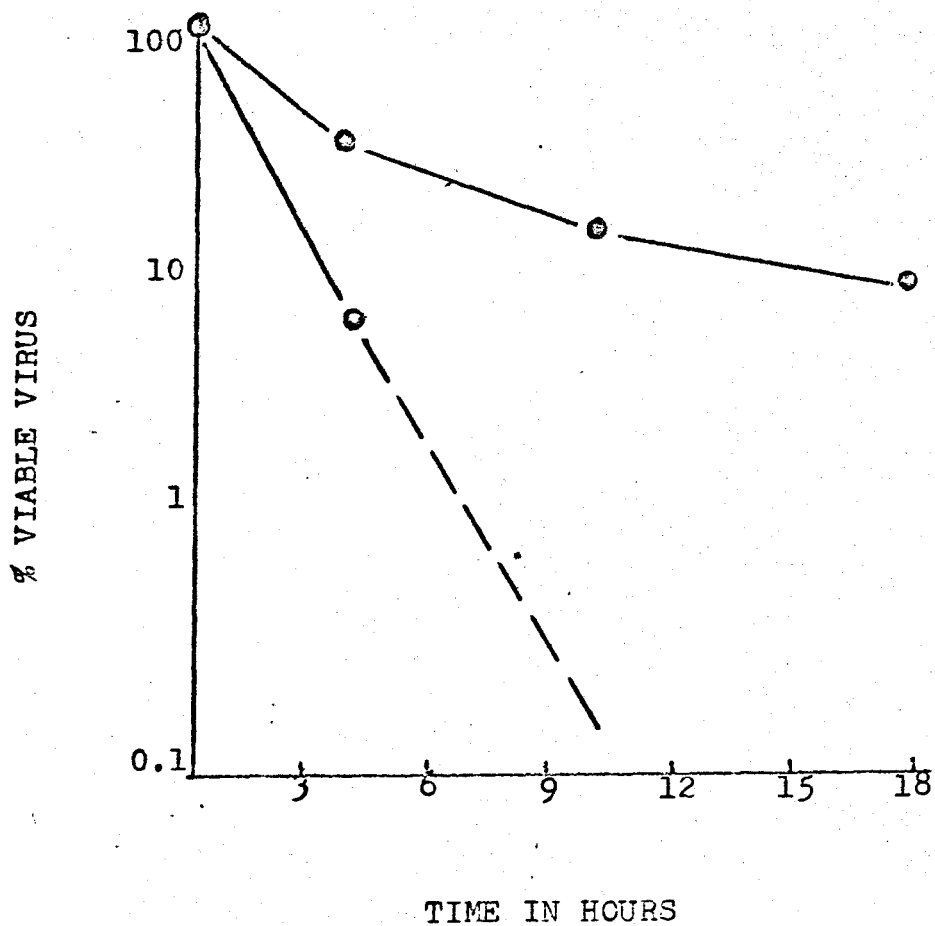


Fig. 5A Inactivation at 37° C of virus from the culture fluid of rabbit kidney cells.

●—● = Type 1 virus

○—○ = Type 2 virus

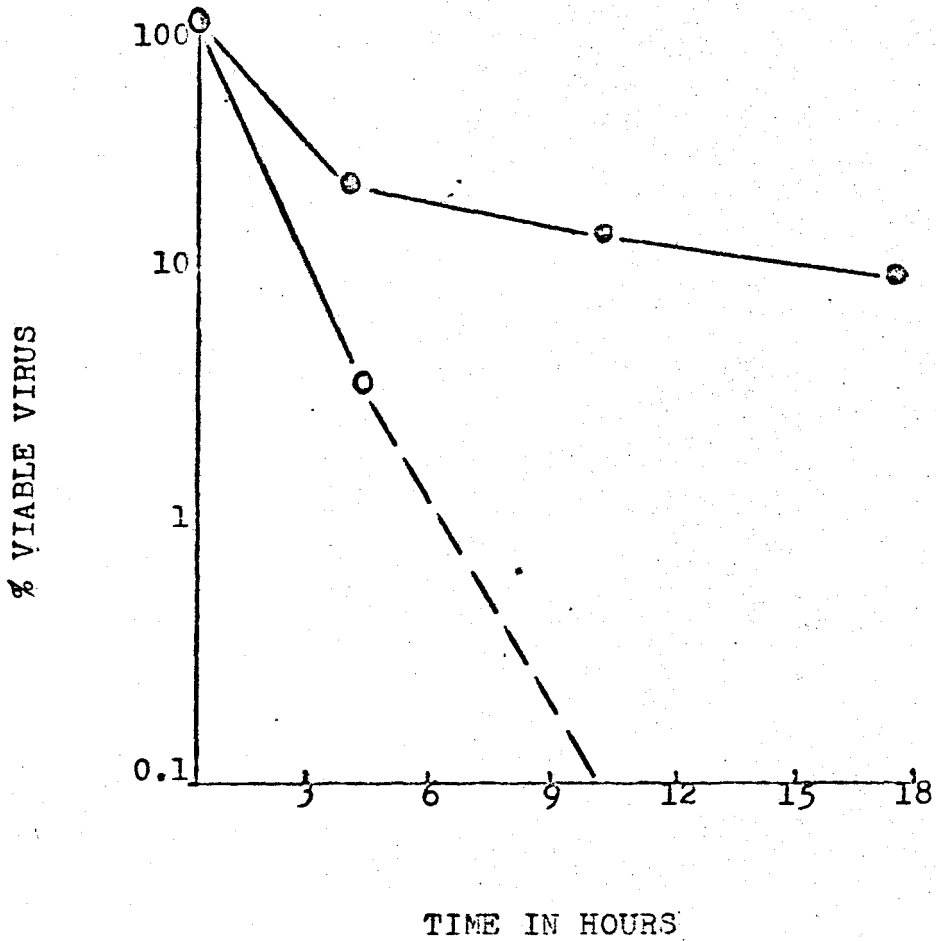


Fig. 5B Inactivation at 37° C of cell associated virus from rabbit kidney cultures.

●—● = Type 1 virus

○—○ = Type 2 virus

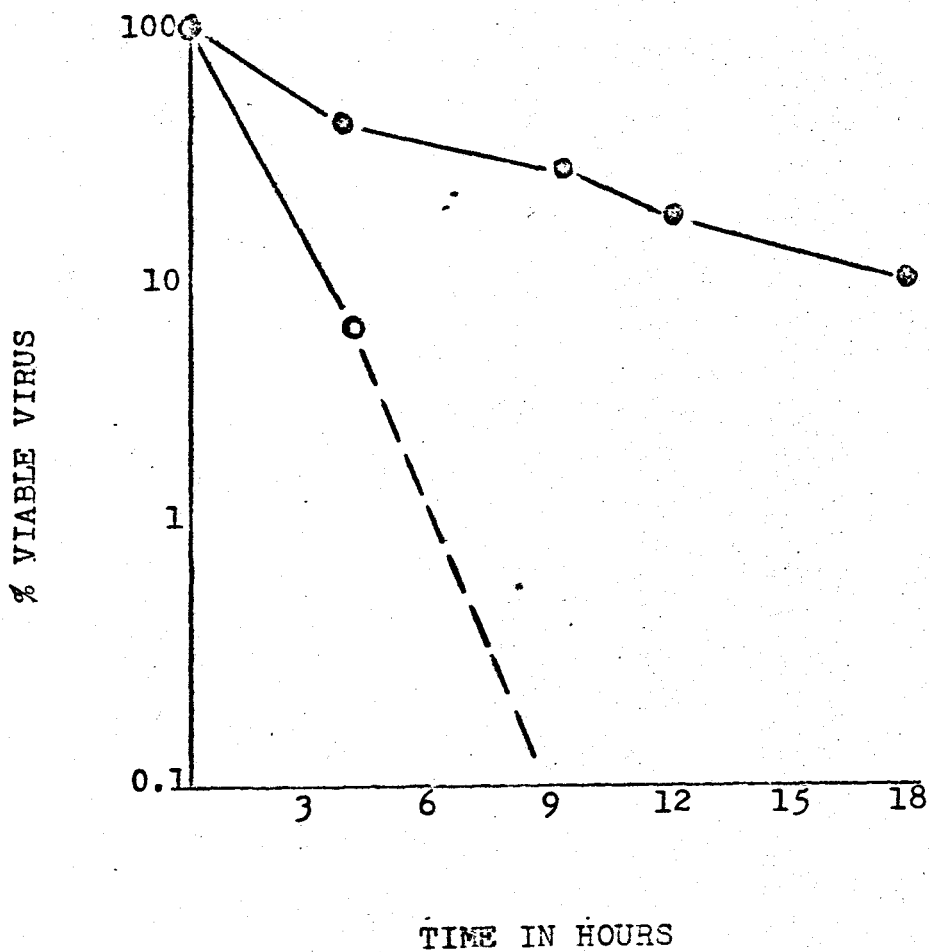


Fig. 5C Inactivation at 37° C of virus from the culture fluid of mouse fibroblasts.

●—● = Type 1 virus

○—○ = Type 2 virus

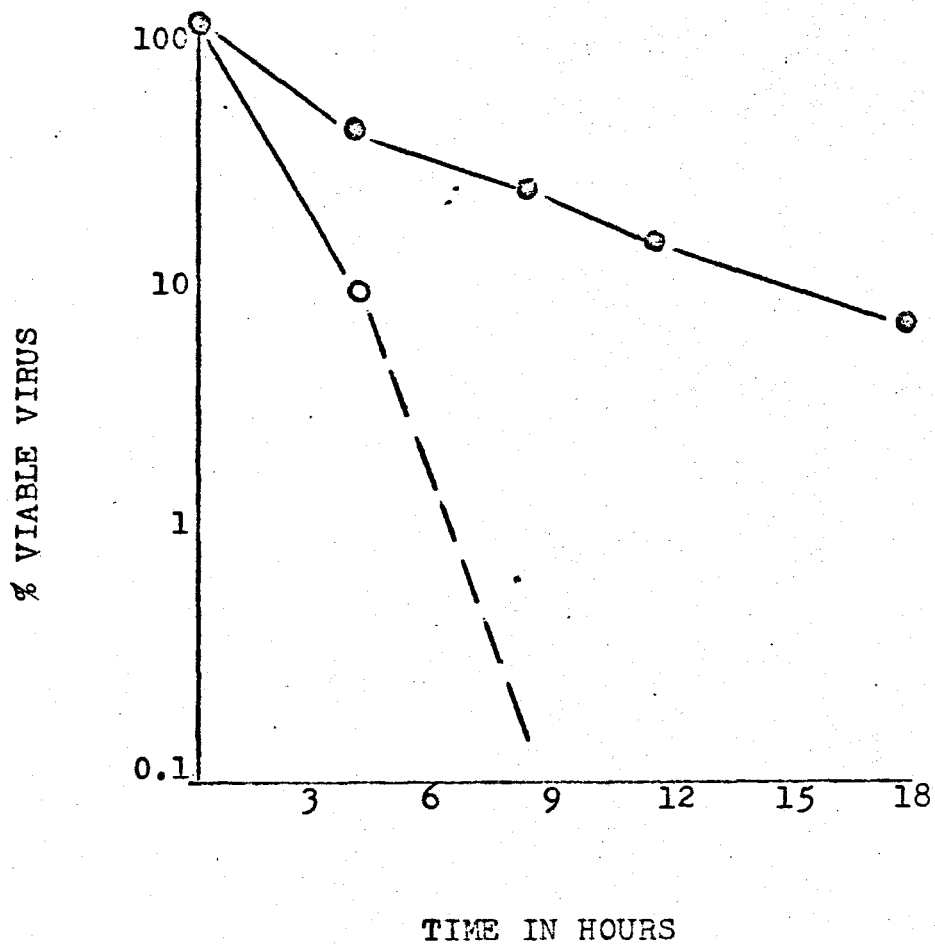


Fig. 5D Inactivation at 37° C of cell associated virus from mouse fibroblasts.

●—● = Type 1 virus

○—○ = Type 2 virus

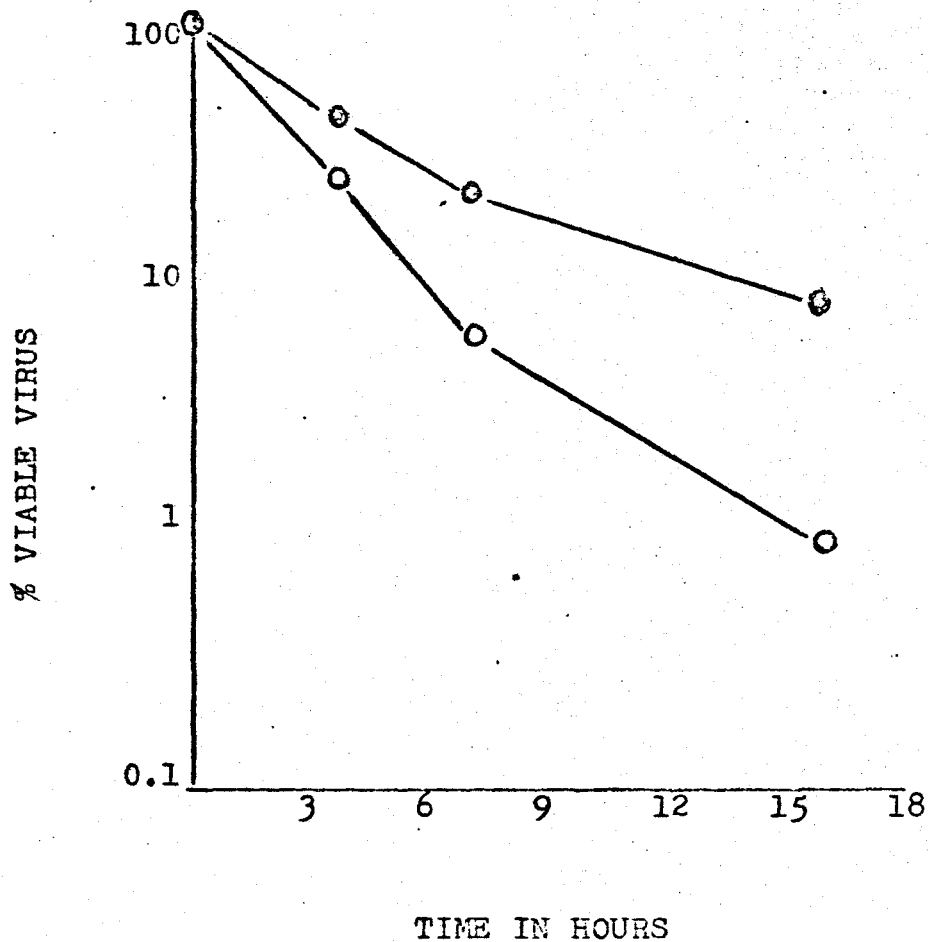


Fig. 5E Inactivation at 37° C of virus from the culture fluid of human fibroblasts.

○—○ = Type 1 virus

●—● = Type 2 virus

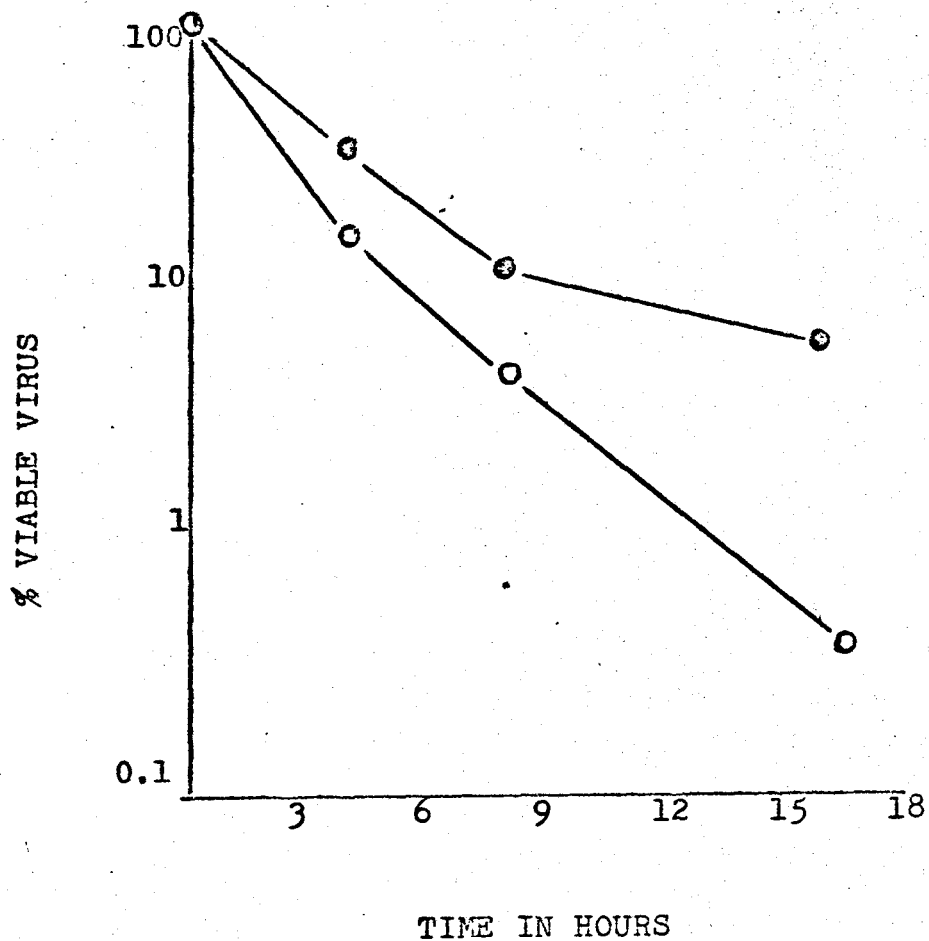


Fig. 5F Inactivation at 37° C of cell associated virus from human fibroblasts.

—○— = Type 1 virus

—○— = Type 2 virus

Periodically samples were removed and titered to measure inactivation.

The time at which virus was collected did not influence the relative stabilities of either type 1 or type 2 virus (Figs. 6A,B). Aliquots of either type stored at -70 C and periodically thawed, titered, and refrozen showed virtually no loss of infectivity over a six month period (Table 2). At 4 C type 2 virus was slightly more unstable than type 1 (Fig. 6C). Inactivation of type 1 and type 2 virus at 37 C resulted in instability curves identical to those presented in Fig. 5A for rabbit kidney culture fluid virus where type 2 was shown to be more unstable than type 1.

Effect of Inactive Virus Particles On Neutralization Curves.

In view of the greater instability of type 2 virus, two experiments were performed to determine if the presence of uninfected particles could interfere with virus neutralization and this explains any inability to detect serological differences with type 2 antiserum. In the first experiment a neutralization curve was done on a strain of type 2 virus diluted 1/10 in 199 diluent and 1/10 in a virus suspension that had been inactivated at 37 C. In the other experiment a neutralization curve was done on a strain of type 2 virus harvested at 24 and 114 hr. The virus suspension taken at 114 hr would have a far greater proportion of inactive particles than the 24 hr harvest.

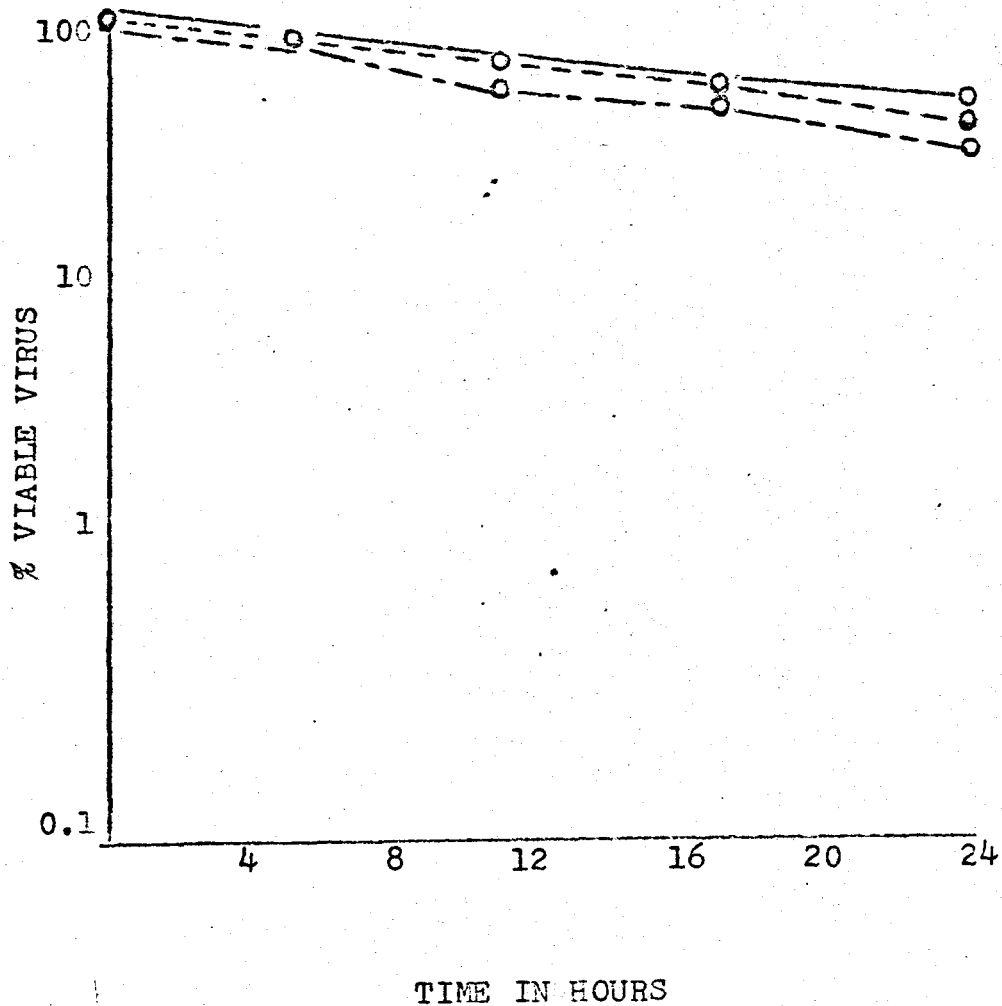


Fig. 6A Inactivation at 4° C of type 1 virus released at different times of the growth cycle.

0—0 = 22-26 hrs.

0-----0 = 46-50 hrs.

0- - - - -0 = 70-74 hrs.

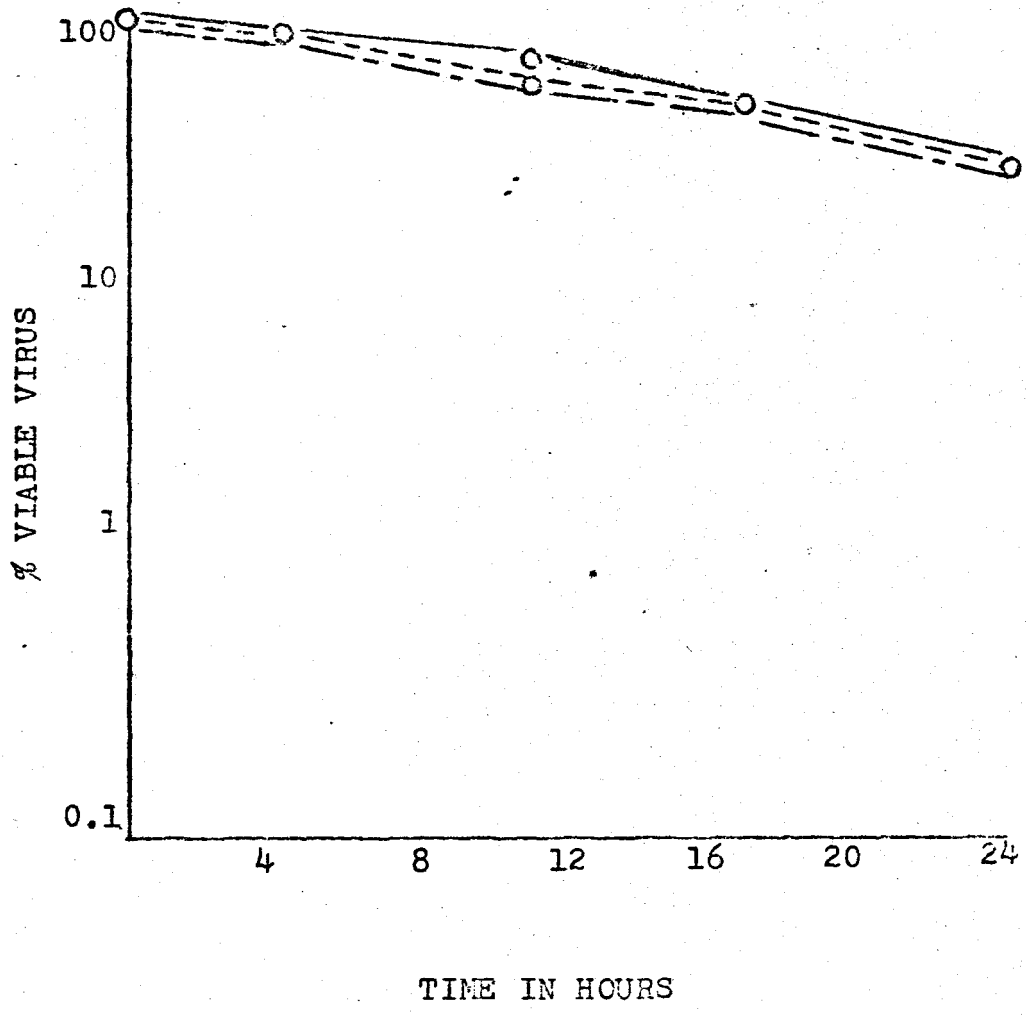


Fig. 6B Inactivation at 4° C of type 2 virus released at different times of the growth cycle.

- 0— — — — — 0 = 22-26 hrs.
- 0- - - - - 0 = 46-50 hrs.
- 0- 0 = 70-74 hrs.

Table 2 The effect of freezing and thawing on type 1 and type 2 viruses harvested at various periods of the growth cycle.

Time of freezing and thawing	Time of virus release					
	22-26 hrs.		46-50 hrs.		70-74 hrs.	
	Type 1	Type 2	Type 1	Type 2	Type 1	Type 2
Initial titer	3.25	1.53	4.75	2.44	4.75	2.70
24 hr.	2.06	1.74	4.70	2.49	4.73	2.83
48 hr.	3.08	1.62	4.76	2.30	4.78	2.76
72 hr.	3.11	1.62	4.68	2.20	4.70	2.55
2 weeks	3.05	1.51	4.65	2.11	4.61	2.40
6 months	3.05	1.55	4.60	2.05	4.65	2.40

Virus titers are given in \log_{10} .

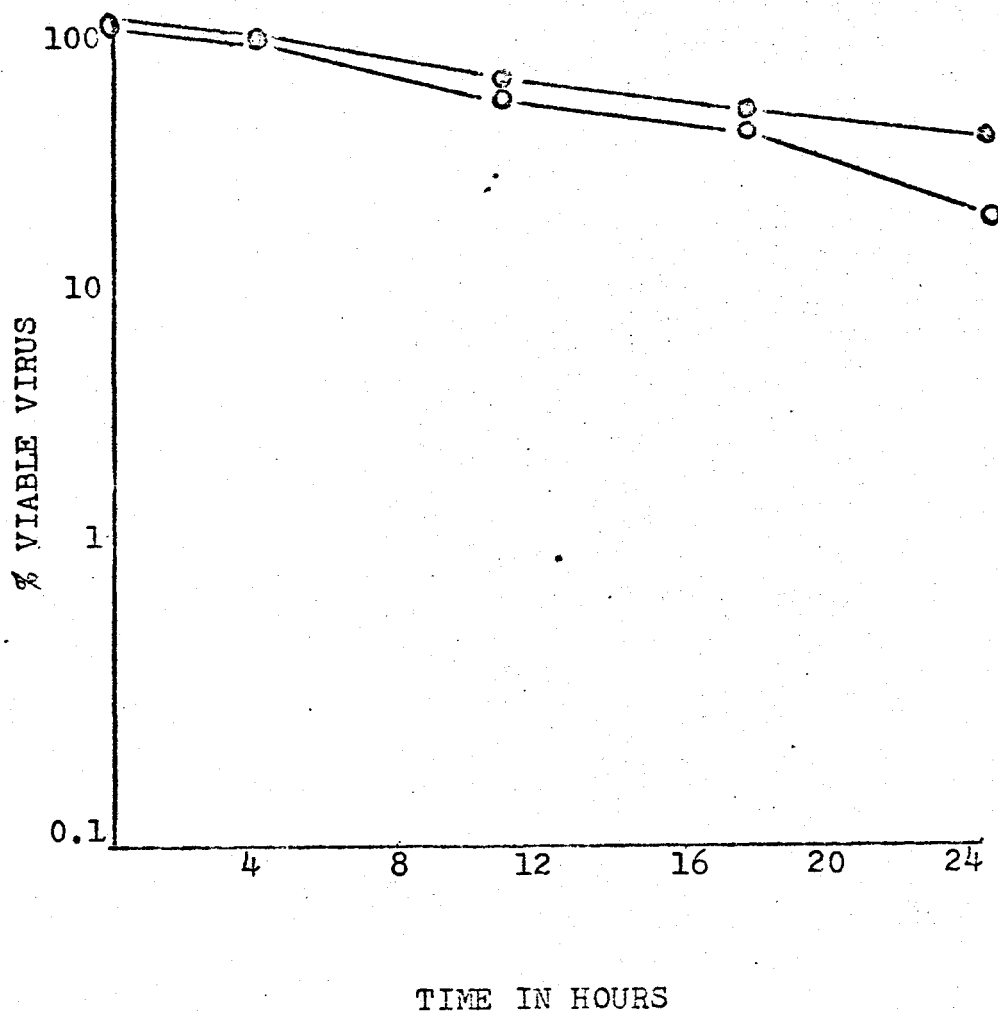


Fig. 6C A comparison at 4° C of the stabilities of type 1 and type 2 viruses.

●—● = Type 1 virus

○—○ = Type 2 virus

The results of these experiments indicate that the presence of inactive virus particles may block neutralization (Figs. 7A,B). The virus harvested at 24 hr or diluent was neutralized more rapidly than the virus harvested at 114 hr or diluted in inactivated virus suspension.

Sensitivity of Type 1 and Type 2 Viruses to Heparin

The effect of heparin on herpes simplex types 1 and 2 was investigated because of a report (Lehel and Hadhazy, 1966) indicating that type 2 strains were less sensitive to its effect. Heparin (Abbott, North Chicago, Ill.) at a concentration of 100 USP units/ml was added to the media of rabbit kidney tissue cultures one hr before the cultures were inoculated with 1000 pfu of virus. The cell sheet was observed for several days for the appearance of cytopathic effect. Figure 7 compares the appearance of CPE in control tubes without heparin to those with it. It is evident that type 2 viruses are less sensitive to heparin's effect than type 1 strains.

DNA Density of Type 1 and Type 2

Scanner tracings of DNA from rabbit kidney cells infected with either type 1 or type 2 virus and centrifuged to equilibrium in cesium chloride are shown in Figs. 8A and B, respectively. Each of the type 1 strains tested had a density of 1.727g/ml and each type 2 strain had a density of 1.729g/ml.

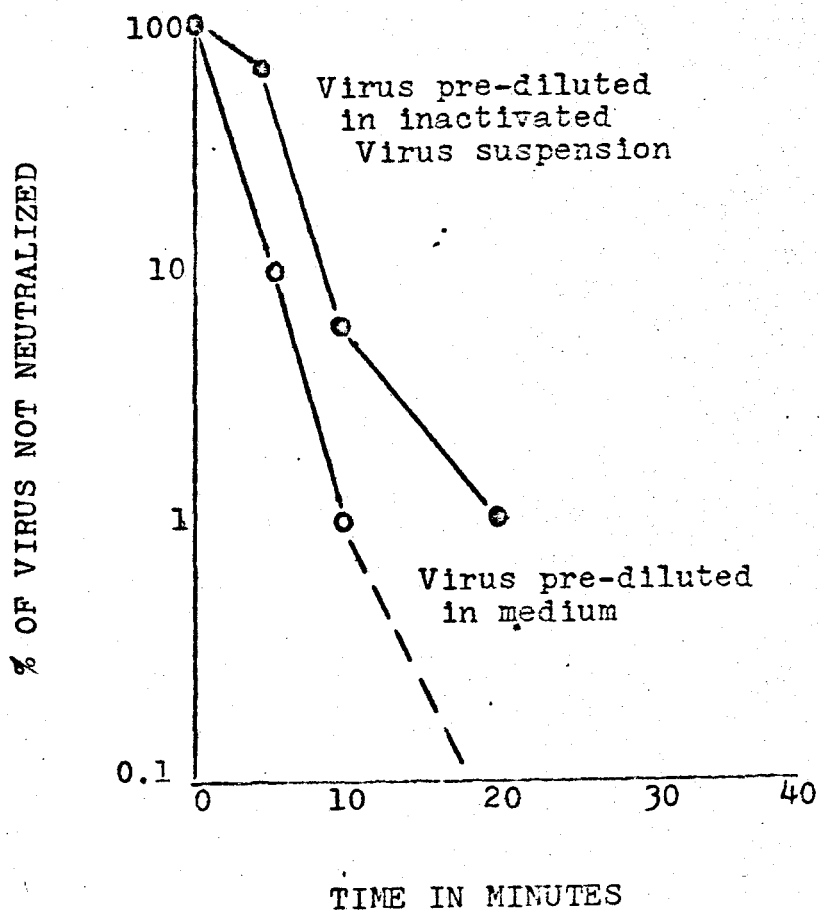


Fig. 7A Neutralization of type 2 virus prediluted in medium and in inactivated virus suspension.

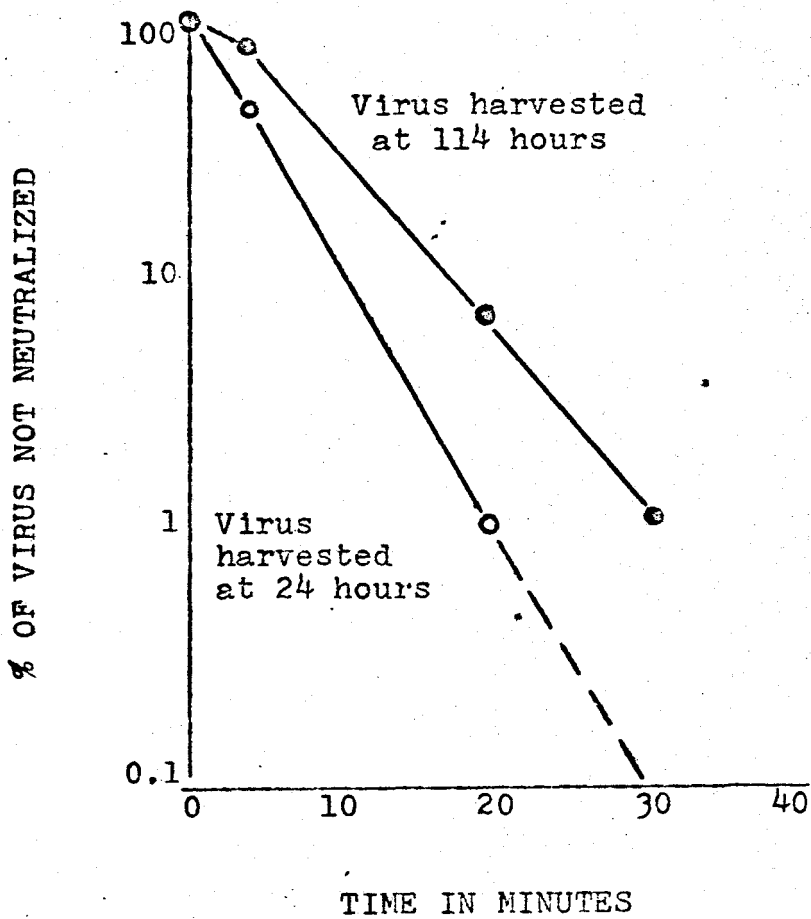


Fig. 7B Neutralization of type 2 virus harvested at 24 and 114 hours.

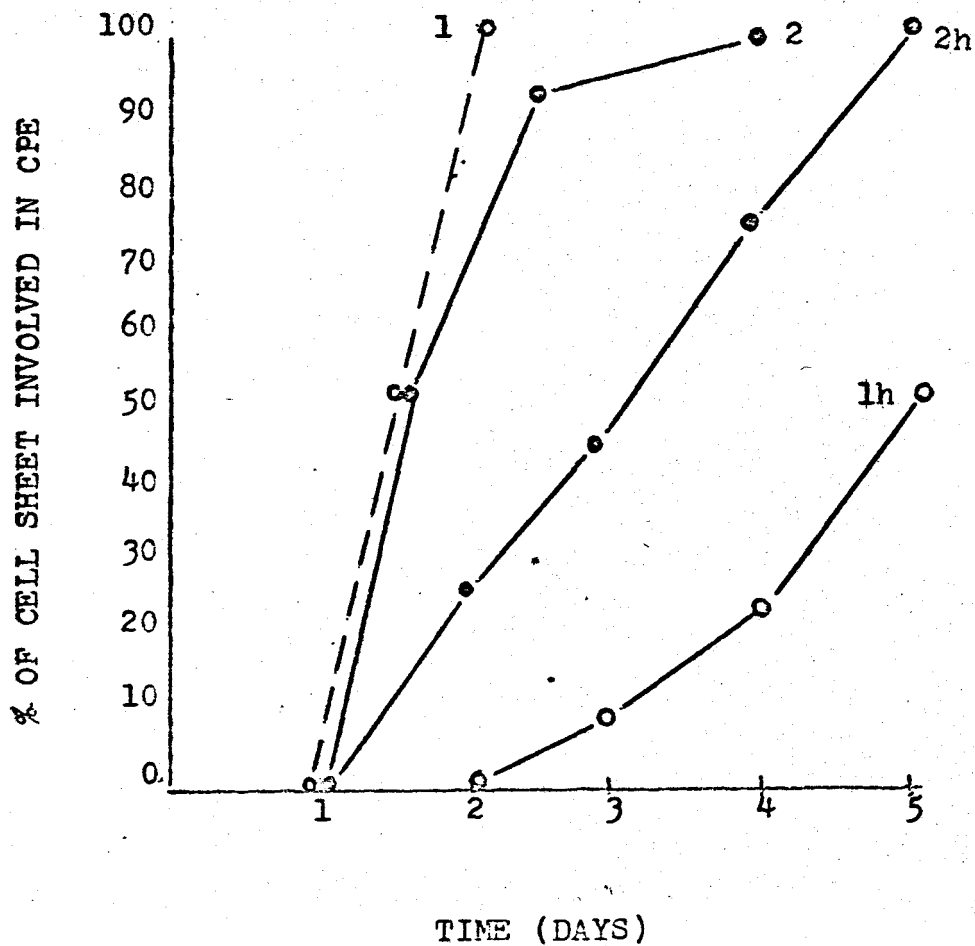


Fig. 12 The inhibitory effect of heparin on type 1 and type 2 viruses.

1 = Type 1 virus in the absence of heparin

1h = Type 1 virus + heparin

2 = Type 2 virus in the absence of heparin

2h = Type 2 virus + heparin

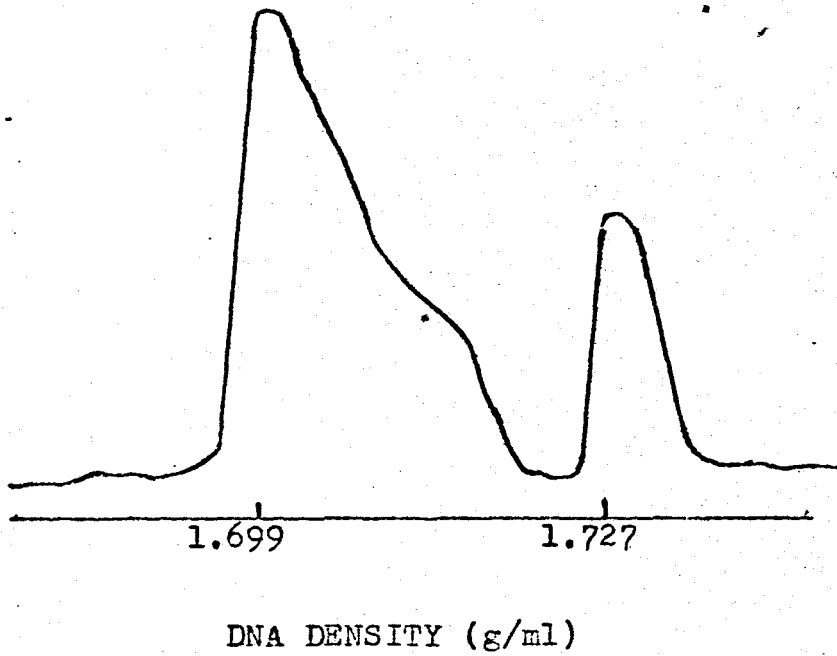
ABSORPTION AT 265 m μ 

Fig. 8A Scanner tracings taken at 265 m μ of DNA extracted from rabbit kidney cells infected with type 1 virus.

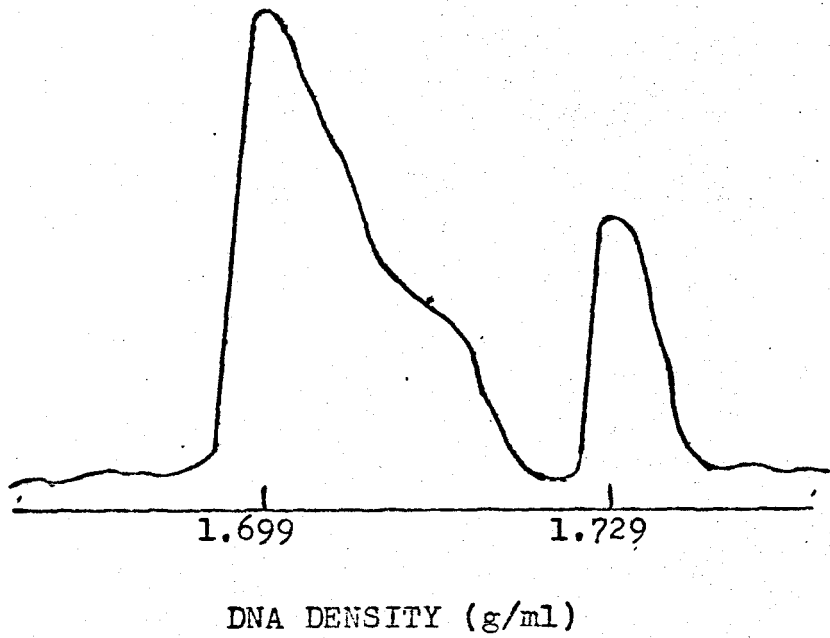
ABSORPTION AT 265 m μ 

Fig.8B Scanner tracings taken at 265 m μ of DNA extracted from rabbit kidney cells infected with type 2 virus.

These readings correspond to base compositions of 68.3% G+C content for type 1 and 70.4% G+C content for type 2. In each figure the left peak is rabbit cell DNA(1.699g/ml). The figure for rabbit DNA was also determined in a separate run by mixing it with clostridium perfringens DNA(1.691g/ml). This was done to insure that the relative position of cell DNA was correct. A mixture was made of the DNA's of each virus type and a separate run made to be certain that the density difference of the two viruses was real. Figure 8C shows that the two viral peaks are clearly resolved when mixed together.

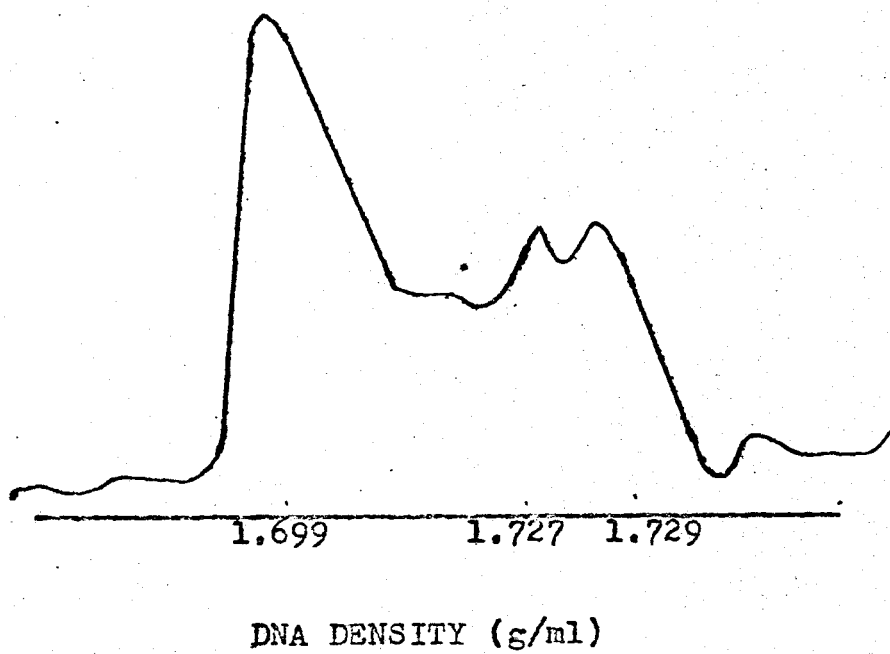
ABSORPTION AT 265 m μ 

Fig. 8C Scanner tracings taken at 265 m μ of cell DNA and mixed type 1 and type 2 viral DNA.

CHAPTER V

Results-Biological Studies

Plaque Morphology

During the course experimentation involving herpes simplex viruses, it became apparent that there was a considerable difference in the appearance of plaques formed by type 1 and 2. In fact, it is possible, based on plaque morphology alone, for someone with little experience in viewing viral plaques to distinguish between plaque type 1 and type 2 strains in rabbit kidney cells. A photomicrograph of a typical type 1 plaque in rabbit kidney tissue culture appears in plate 1. A type 2 plaque in rabbit kidney culture is presented in plate 2. Type 2 strains always produce plaques that are more easily seen and distinguishable than type 1 strains. Plaques from type 2 strains invariably have a higher ratio of giant cells to small round cells than do type 1 plaques. The giant cells associate with aggregates of smaller round cells which often pile up into clumps. These clumps after pull away from the center of infection forming a clear area. Type 1 strains produce foci of small round cells which may clump together in a pock-like shape. They rarely contain a clear area and usually possess an irregular periphery



Plate 1 A herpes simplex type 1 plaque in rabbit kidney tissue culture.

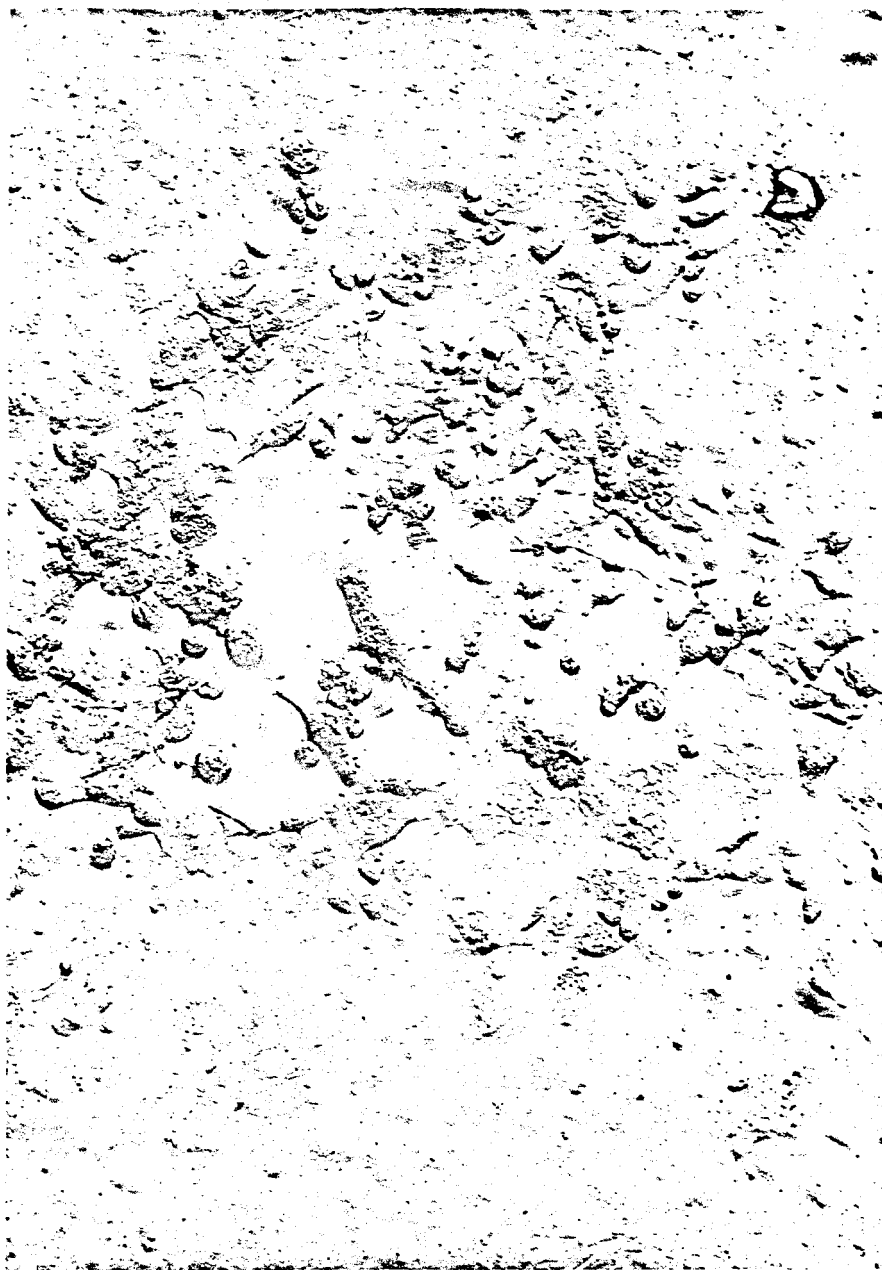


Plate 2. A herpes simplex type 2 plaque in rabbit kidney tissue culture.

of arm-like extensions containing small round cells. Type 2 plaques in contrast, possess a more regular periphery, usually bordered by many more giant cells than are type 1 plaques.

Growth Studies of Types 1 and 2 In Rabbit Kidney, Mouse Embryo and Human Fibroblast Cultures

Strains of type 1 virus consistently produced higher titers than did type 2 strains in rabbit kidney cultures, mouse embryo cultures and human fibroblasts. The degree of difference was about one log when human fibroblasts served as host whereas it was 2 to 3 logs with either of the other cultures. In spite of the differences in the ultimate titers achieved, the length of the eclipse periods was the same, 10 hr, for both virus types in each of the cell cultures. The length of the latent periods varied however. Table 3 summarizes the important growth characteristics of the viruses in these three cell systems and Figs. 9A,B,C present intracellular and extracellular growth curves of both virus types in each of the tissue cultures.

In order to test the hypothesis that the smaller titers produced by type 2 strains were due to the virus' instability, intracellular growth curves of type 1 and type 2 strains were done by incorporating into the overlay medium neutralizing antibody. This prevented infection of surrounding cells through the fluid medium and negated any growth advantage that type 1 strains might possess due to an increased capability of infect-

TABLE 3. Behavior of Type 1 and Type 2 Herpes Simplex
viruses in different tissue culture systems

	<u>Length of eclipse period (in hours)</u>		<u>Length of latent period (in hours)</u>		<u>Maximum titers of stock virus attained per ml of culture fluid (log₁₀)</u>	
	<u>Type 1</u>	<u>Type 2</u>	<u>Type 1</u>	<u>Type 2</u>	<u>Type 1</u>	<u>Type 2</u>
Rabbit kidney cultures	10	10	15	19	6-7	2-4
Human fibroblast cultures	10	10	19	22	6-7	4-5
Mouse embryo cultures	10	10	16	20	4-5	2-3

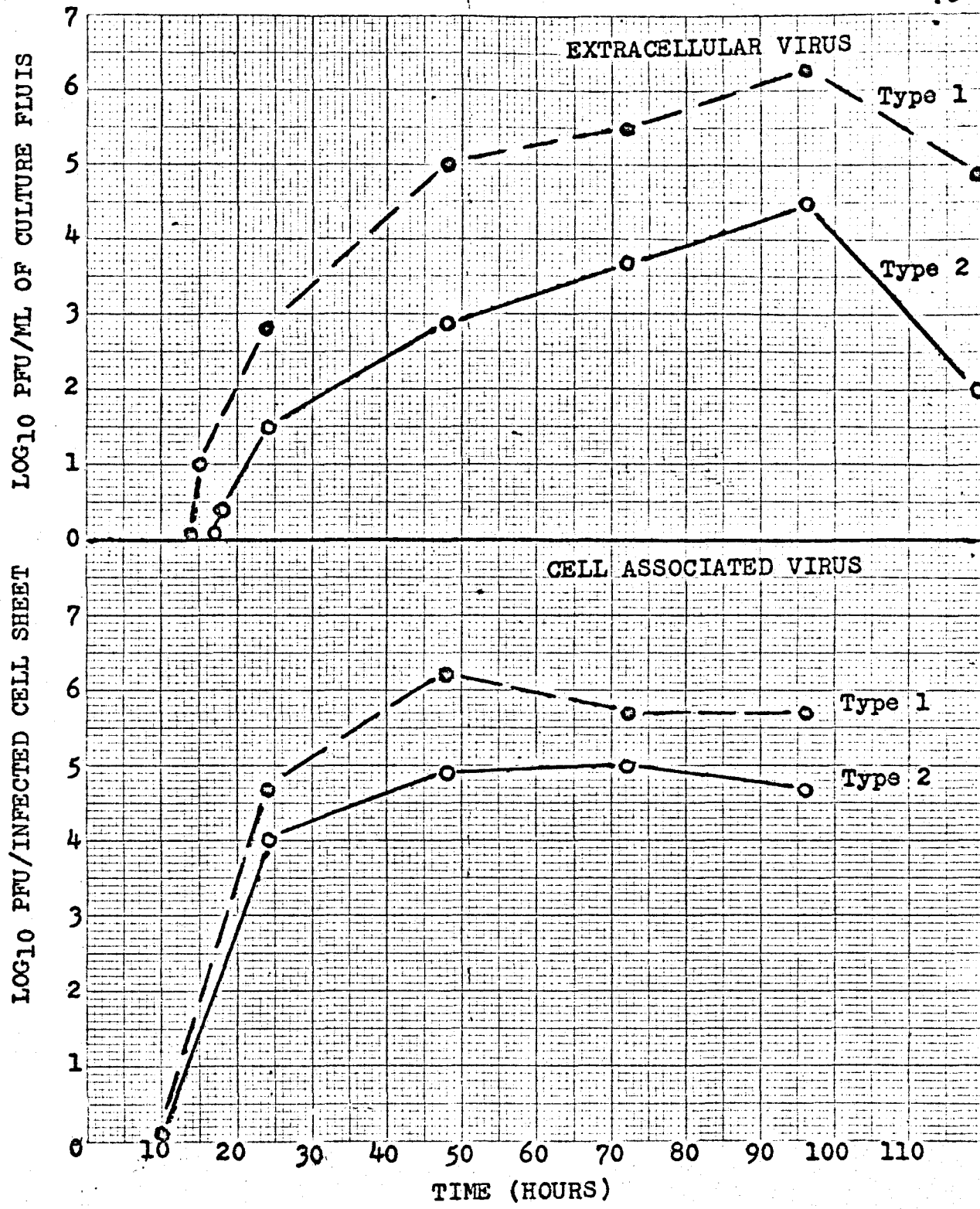


Fig. 9A Comparative growth curves of type 1 and type 2 viruses in rabbit kidney tissue culture

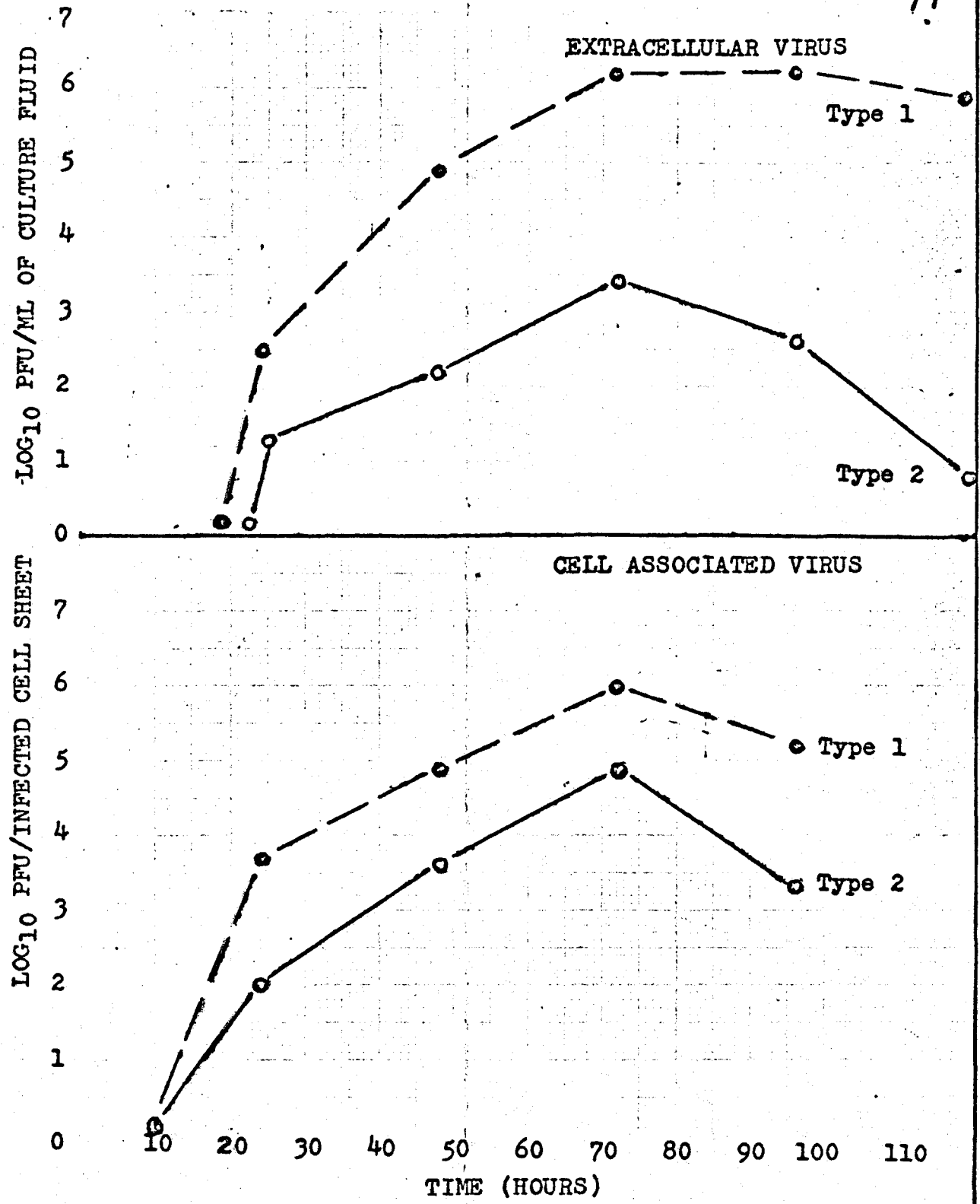


Fig. 9B Comparative growth curves of type 1 and type 2 viruses in human fibroblasts

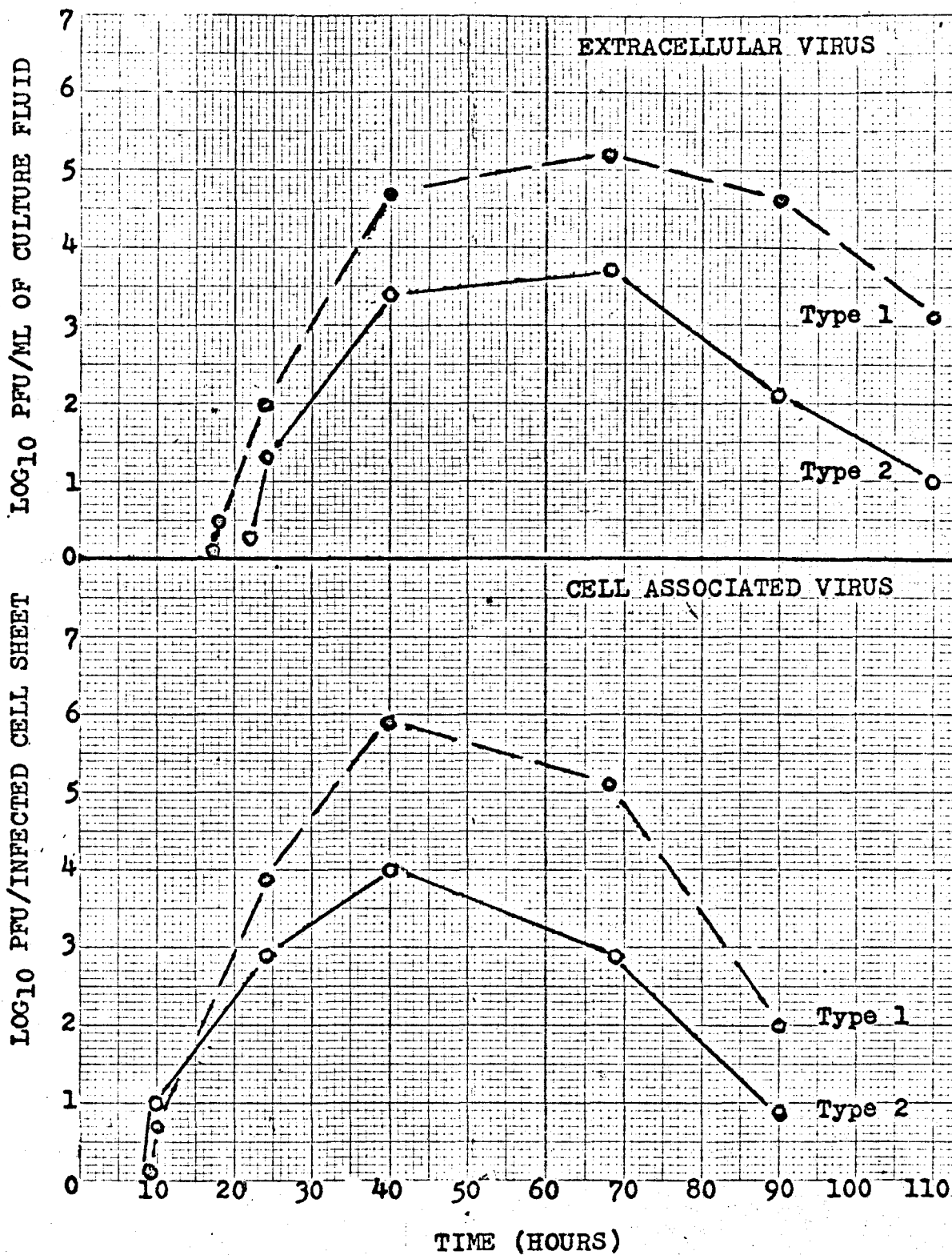


Fig. 9C Comparative growth curves of type 1 and type 2 viruses in mouse embryo tissue culture

ing more cells merely because more infectious particles are available through their greater stability. The actual experiment was done in the same way as the other growth curve experiments except for the presence of specific antibody. Cells were washed 5 times with 199 diluent before they were disrupted to eliminate antibody at the time of assay. Fig. 10 shows that although the initial growth was the same for both virus types, the ultimate titer achieved for type 1 was still greater than for type 2.

Neurovirulence Experiments

All experiments involving the relative neurovirulence properties of herpes simplex types 1 and 2 indicate that type 2 strains are more neurovirulent. None of the five different type 1 strains tested could induce paralysis or myelitis in 5 month old rabbits whereas all five type 2 strains tested did demonstrate such an effect (Table 4). It is of further interest that inoculum used for injection of 3 of the type 1 strains was 2 logs higher than any of the type 2 strains tested. Histological examination of rabbits paralysed by type 2 virus showed inflammation of dorsal ganglia and horns.

In mice only 4 of 92 animals injected with type 1 strains showed CNS involvement whereas 41 of 88 mice inoculated with type 2 strains showed CNS involvement (Table 5).

Parallel titrations of type 1 and type 2 virus strains in

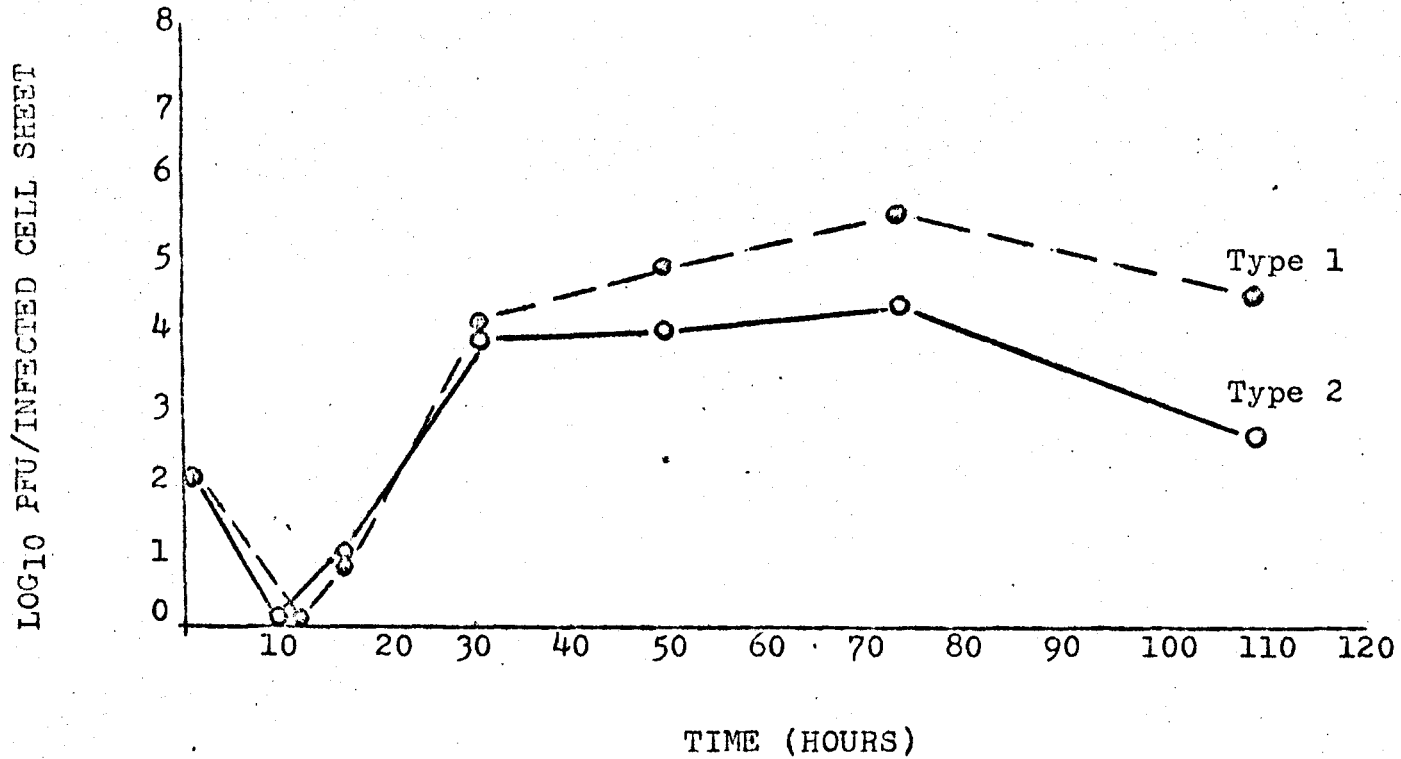


Fig. 10 Comparative intracellular growth curves of type 1 and type 2 viruses in rabbit kidney tissue culture.

Table 4 Neurotropic Properties in Adult Rabbits of Type 1 and Type 2 Strains of "Herpes Simplex Viruses"

<u>TYPE 1</u>			<u>TYPE 2</u>		
<u>Strain</u>	<u>Titer of Inoculum</u>	<u>Numbers developing paralysis</u>	<u>Strain</u>	<u>Titer of Inoculum</u>	<u>Numbers developing paralysis</u>
Watson	10^4	0/5	Wiggins	10^4	6/11
Watson	10^6	0/6	D64	10^4	3/10
197	10^4	0/5	Dawson	10^4	3/10
197	10^6	0/6	US	10^4	7/10
356	10^4	0/6	MS	10^4	6/10
L2	10^6	0/10			
BW	10^6	0/11			

T. Value 7.1

TABLE 5

Neurotropic Properties of Type 1 and Type 2 strains in mice indicated as numbers of inoculated mice showing CNS involvement.

Type 1		Type 2	
197	0/36	WIGGINS	13/35
WATSON	3/34	DAWSON	16/33
L2	1/22	MS	12/20
T = 5.2			

three different tissue culture systems and intracerebrally in suckling mice indicate greater neurovirulence properties for type 2 virus (Table 6). Among the seven type 1 strains tested only one showed a higher LD₅₀ to pfu ratio whereas none of 7 type 2 strains showed a ratio of LD₅₀ to pfu less than 1:1. The titrations in mouse embryo tissue culture resulted in every instance in values less than those obtained in rabbit kidney cells indicating that neither herpes simplex virus type grows preferentially in mouse tissue.

TABLE 6. Parallel titrations of Type 1 and Type 2 isolates in three different tissue cultures systems and intracerebrally in suckling mice. The four titers in each horizontal column represent the amount of virus per 0.2 ml of a given stock of each strain as measured by the different systems.

Virus Type 1	Tissue Culture	PFU (Log ₁₀)				Ratio: LD ₅₀ titer	Rabbit cell PFU titer
		LD ₅₀ in mice	Rabbit kidney cultures	Human fibroblast cultures	Mouse embryo cultures		
197	4	3.6	5.0	5.1	4.4	1:25	
L2	20?	5.8	6.0	ND	5.4	1:2	
356	4	4.7	4.2	ND	3.6	3:1	
Watson	4	1.5	4.0	3.9	3.7	1:316	
Glover	4	4.7	5.1	5.0	4.6	1:3	
E515	4	4.3	4.7	4.7	4.2	1:3	
Slatton	4	5.0	5.3	5.3	4.5	1:2	
<u>Type 2</u>							
ST1	2	4.4	4.0	4.1	3.2	3:1	
ST2	2	4.9	4.3	4.3	3.7	4:1	
Wiggins	4	3.8	3.0	ND	ND	6:1	
Dawson	4	2.9	3.0	2.9	2.2	1:1	
MS	10	4.0	3.0	3.0	2.7	10:1	
D64	5	1.9	1.9	1.9	1.2	1:1	
Willis	4	4.0	3.2	3.2	2.6	6:1	

CHAPTER VI

Isolation and Characterization of A Herpes Simplex Large Plaque Variant

In the course of passing the herpes simplex type 1-Watson strain through baby mouse brain through repeated inoculations a variant was isolated at the seventh passage. It was characterized by its ability to form larger plaques than its parent strain which were syncytial in appearance. Upon isolation and plaque purification this variant was found to be stable through five passages in rabbit kidney tissue culture. Neutralization curves identified it as type 1 but it was not neutralized as well by type 2 antiserum as its parent strain (Fig. 11). This indicated a serological change, probably a loss of some of the antigens possessed by the parent strain that are neutralized by type 2 antiserum. Stability curves of the type described previously showed no difference between the large plaque variant and its typical type 1 parent strain. The density of the variant's DNA was found to be 1.727g/ml which was identical to that found for type 1 strains (Fig. 8A).

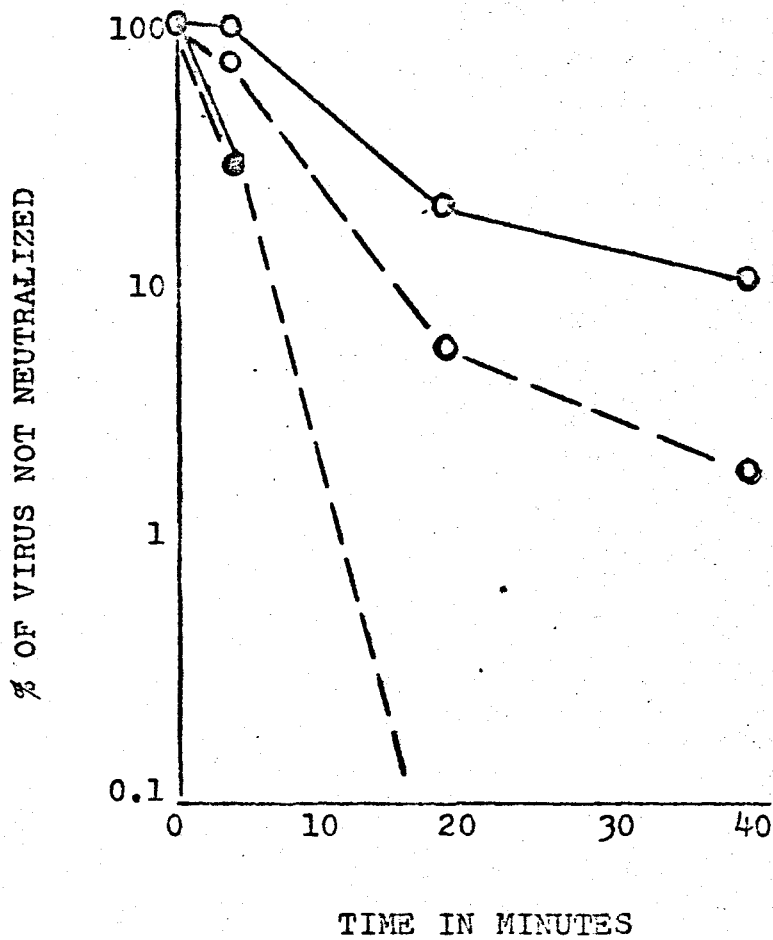


Fig. 11 Neutralization of a large plaque variant by rabbit type 1 and type 2 antiserum.

○—○ = Type 1 antiserum

○—○ = Type 2 antiserum

— — — = Watson large plaque variant

— — — = Watson parent strain

CHAPTER VII

DISCUSSION

There are two types of herpes simplex virus that can be distinguished serologically (Schneweis, 1962; Plummer, 1964; Pauls and Dowdle, 1967) and are associated with their sites of isolation (Dowdle et al., 1967). Type 1 strains are recovered exclusively from non-genital areas of the body and type 2 strains are associated only with the genitalia. This knowledge permitted isolation of many strains of each type with which to study comparative serological, physical and biological characteristics. Ten type 1 strains and thirteen type 2 strains were identified by means of neutralization curves and a perfect correlation was found between their sites of isolation and their type specificity. Seven of the type 1 strains and eight of the type 2 strains had multiple passage histories in rabbit kidney tissue culture, yet they were as easily identified in our hands as the three type 1 and five type 2 strains that were tested after a single passage in rabbit kidney tissue culture. This observation together with a controlled experiment where no change in serologic specificity occurred after thirty passages in rabbit kidney tissue culture of three type 1 and

three type 2 strains strongly indicates that the type specificity is a stable characteristic.

The serologic difference between type 1 and type 2 viruses was demonstrated with antiserum to either type when the viruses were grown in rabbit kidney tissue culture. Schneeweis (1962) and Pauls and Dowdle (1967) were able to distinguish the types only with type 1 antiserum. The occurrence of large amounts of inactive particles in type 2 virus stocks can interfere with viral neutralization (Figs. 7A,B) and it is possible that this is the reason why the above workers were unable to detect type differences between type 1 and type 2 strains using type 2 antiserum. It should be noted, however, that the procedure used by Pauls and Dowdle (1967) for the preparation of specific antiserum was identical to that used in our laboratory. The specificity of the sera used in each laboratory would be expected to be the same. Their inability to detect type differences with type 2 antiserum, therefore, may result from technical differences in the neutralization test. They employed a micro-neutralization technique and used secondary rabbit kidney cells in their assay, while all of our tests involved primary rabbit kidney cells. The extra passage of the tissue culture may involve a change in the configuration of viral receptors on the cell surface resulting in differences in the virus assay.

The serologic difference between herpes simplex types 1 and

2 can be readily detected using type 1 antiserum when the viruses are grown in rabbit kidney, mouse embryo and human fibroblast tissue cultures. The type specificity is less easily recognized when 1 and 2 are grown in mouse and rabbit brain and type 1 antiserum is used for identification. Type 2 antiserum readily distinguished between types 1 and 2 when they were grown in rabbit kidney and mouse embryo tissue culture. Virus grown in human fibroblasts was less easily identified while virus grown in mouse and rabbit brain could not be identified as to type specificity with type 2 antiserum. Even type 1 antiserum did not distinguish easily between type 1 and type 2 viruses grown in mouse and rabbit brain. These serologic studies indicated that type 2 virus changes antigenically to some degree when grown in various cell systems. The antigenic sites that change must be related to the virion's infective capability since our neutralization test was biological in nature and dependent upon an ability to measure infectious particles. The envelope surrounding herpes simplex particles is acquired from host cell membranes (Darlington, 1968) and appears to be required for infectivity (Spring and Roizman, 1968). It is reasonable to assume that the antigenic sites which change on type 2 viruses are on the envelope. This phenomenon of different antigenic determinants is probably not restricted to type 2 virus. A careful comparison of Figs. 1A and 1B with Figs. 1D

and 1E reveals that type 1 virus was neutralized to a smaller extent with type 1 antiserum when grown in mouse and rabbit brain than when grown in rabbit kidney and mouse embryo tissue culture. Structures on the envelope which are usually recognized as type 2 determinants are either masked by changes in the envelope's configuration or are not present at all when type 2 antiserum fails to distinguish between its homologous virus and a type 1 strain. The presence or absence of antigenic sites is dependant upon the cell system in which the viruses are grown and is demonstrated in the ability of antiserum to recognize them. In rabbit kidney and mouse embryo tissue culture recognition is easy, but in rabbit and mouse brain it is difficult or impossible. A viral change in antigenicity due to growth in different cell systems with subsequent differences in envelopment is not a new finding in virology. Cartwright and Pearce (1967) reported antigenic differences within the envelope of vesicular stomatitis virus grown in baby hamster kidney cells and pig kidney cells. Virus grown in baby hamster kidney cells possessed a different protein component in the envelope than virus grown in pig kidney cells.

It has been suggested that because the envelope consists of viral antigenic determinants and components specified by the cells there may be a correlation between the CPE of infected

cells and selected properties of the virus (Ejercito et al., 1968). Our comparative study of types 1 and 2 support this suggestion. Type 1 and type 2 viruses differ antigenically, probably because of different antigenic determinants in the envelope. They also differed in the CPE each produced in rabbit kidney tissue culture. Since cellular membranes must be modified by products specified by the virus for the virion to become enveloped, then the viral product binding to the membranes is responsible for the alteration in the cell membrane resulting in the characteristic viral CPE of infected cells.

Two other differences between type 1 and type 2 viruses that may involve the envelope were; 1) the relative stabilities of each type and 2) the ultimate titer of infectious particles achieved in tissue culture by each type. Type 2 viruses were more unstable at 37°C than type 1 virus in rabbit kidney and mouse embryo tissue cultures while type 1 strains consistently produced 2-3 logs more virus when grown in these cultures than type 2 strains. In human fibroblasts, however, type 2 virus was more stable than in either rabbit kidney or mouse embryo cultures and reached higher titers of infectious particles. Both virus types had identical eclipse periods indicating that the same amount of time was required for both types to synthesize an infectious particle. The latent periods of both virus types varied between each cell system but, in any given cell culture

type 1 virus always appeared in the culture fluid before type 2. This may mean either that the envelopment and release of type 1 particles was more efficient and faster or that because of the greater instability of type 2 virus, it could not be detected until a large number of particles were released. In fact, the difference in latent periods between type 1 and type 2 viruses was least in human fibroblasts where type 2 strains had been shown to be most stable. An experiment designed to account for smaller type 2 virus yields in tissue culture through correlation with instability by incorporating neutralizing antibody into the overlay medium proved inconclusive. Initially, the intracellular growth of both types progressed at about the same pace but eventually the titers of type 1 exceeded those of type 2 (Fig. 10). The effect of the greater instability of type 2 cannot be dismissed as cause of the lower titers. Instability curves of intracellular rabbit kidney virus showed that type 2 was more unstable than type 1 (Fig. 5B). Thus, the very effect that we hoped to eliminate by using antibody in the culture fluid was still present, namely, that there were more viable type 1 particles available for infection of neighboring cells than type 2 particles. It might be suggested that this was irrelevant at the cellular level where there was direct physical contact between cells. It has been demonstrated, however, that by increasing the ratio of

pfu/cell at the time of inoculation, the length of time required for synthesis of infectious particles was shortened (Roizman et al., 1963). In addition, due to the unstable nature of type 2 virus particles, a portion of them may become inactive during the assay procedure and never be counted.

Another indication of the difference between type 1 and type 2 virus envelopes was found in the inhibitory action of heparin, a negatively charged polysaccharide which inhibited type 1 virus more than type 2 (Fig. 12). Other workers (Hochberg and Becker, 1968) have shown that the inhibitory effect was due to interference with attachment of the virus particle to the host cell. The same effect had been reported on E-virus, another herpes virus, by Benda (1966). Viral attachment to a host cell was a function of the virion's envelope and the host cell's membrane. The fact that heparin interfered more with this function in relation to type 1 viruses indicated a structural and/or compositional difference between the envelopes of types 1 and 2.

Vonka and Benyesh-Melnick (1966) reported that a different herpes virus, human cytomegalovirus, was more labile at 4° C than at 37° C when harvested early. Virus harvested later, however, was more labile at the higher temperature. It has been proposed that stability of herpes simplex virus was dependent upon the site within the infected cell where the particle

acquired its envelope (Spring and Roizman, 1968). Acquisition of an envelope appeared to be dependent upon the post-inoculation that the complete virion eluted from the cell. The earliest particles released possessed envelopes acquired from the nuclear membrane and those released later often possessed envelopes derived from various cytoplasmic membranes (Darlington, 1968). In this study attempts to test stability of virus released at different times post-inoculation did not reveal any differences in stability that could be related to the time of viral release. A far greater stability of both virus types at 4° C than at 37° C and virtually no loss in titer of either type when stored at -70° C was demonstrated (Table 2).

Genetic substantiation of type differences between the two herpes simplex viruses was provided from the density studies of the two virus' DNA's. They differed in density by 0.002g/ml and probably in base composition (C + G%) by 2% (Fig. A,B,C). The latter figure was based on the assumption that no unusual bases existed in the viral genome. A difference of 2% in the base ratio adequately accounts for differences in phenotypic expression by herpes simplex types 1 and 2.

The difference between the virus assay in tissue culture and the assay employing mice may account for the apparent greater neurovirulence of type 2 strains. Many of the defective type 2

particles that do not replicate in tissue culture may do so in the animal. Thus, virus particles measured in vivo may not be measured in tissue culture. The ratios of pfu/LD₅₀ that indicated greater neurovirulence of type 2 strains would not be valid if more type 2 particles were measured by means of the LD₅₀ assay than the pfu assay.

If, indeed, type 2 virus was more neurovirulent, it could be due to possession of a greater tropism for nervous tissue or enhanced ability over type 1 strains to grow in nervous tissue. If type 2 strains were more neurovirulent, it would be particularly interesting in view of a recent report associating them with cancer of the cervix (Bawls et al., 1968).

SUMMARY

The identification by means of neutralization curves of 10 type 1 and 13 type 2 strains supported the report of other workers that type 2 strains are recovered primarily from the genitalia whereas type 1 strains are isolated from other areas of the body. The serologic specificity of the two herpes simplex types was a stable characteristic through 30 passages in rabbit kidney tissue culture. Type 1 and type 2 viruses could also be readily distinguished serologically with type 1 antiserum when they were grown in human or mouse fibroblasts. Type 2 antiserum could be used to identify virus grown in mouse fibroblasts but was less effective on virus grown in human fibroblasts. Type 1 and type 2 strains grown in rabbit and mouse brain could be identified using type 1 antiserum although they were indistinguishable with type 2 antiserum.

Strains of type 1 and type 2 viruses could be tentatively identified based upon their plaque morphology in rabbit kidney tissue culture. Type 2 strains consistently produced plaques that were more easily seen and distinguishable than type 1 plaques. This was due primarily to their more regular periphery and greater number of giant cells.

Type 1 strains produced higher titers of infectious virus than type 2 strains when grown in rabbit kidney, mouse embryo and human fibroblast tissue cultures. The length of the eclipse periods was the same for both virus types in each of the cell cultures but the latent periods varied. In addition, type 1 virus grown in each of these cell cultures retained its infectivity longer when held at 37 C than did type 2 virus.

In addition to the phenotypic differences between the two virus types the difference of 2% in G+C% ratio indicated a major genotypic difference.

Based upon experiments conducted in mice and rabbits, type 2 strains appeared to be more neurovirulent than type 1 strains. The defective type 2 particles that do not replicate in tissue culture, however, may do so in the animal. In this case, the apparent greater neurovirulent capacity of type 2 strains would be an experimental artifact due to inoculation of many more type 2 particles than type 1.

A large plaque variant of a herpes simplex type 1 strain was isolated after seven passages of the parent strain through baby mouse brain. The plaque morphology differed from that of type 1 strains in that it was larger and syncytial in nature. Serologically, the variant was neutralized as well as the parent strain by type 1 antiserum but less well than the parent

strain by type 2 antiserum. The G+C% ratio was the same as the type 1 parent.

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APPROVAL SHEET

The dissertation submitted by Joseph Lloyd Waner has been read and approved by members of the Department of Microbiology.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

11/14/69

Date



Signature of Advisor