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ST. LOUIS ENCEPHALITIS VIRUS IN
PRIMARY TISSUE CULTURE.

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ST. LOUIS ENCEPHALITIS VIRUS
IN PRIMARY TISSUE CULTURE

BY

Evelyn B. Wallraff

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1961
I hereby recommend that this dissertation prepared under my direction by Evelyn B. Wallraff entitled St. Louis Encephalitis Virus in Primary Tissue Culture be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy.

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*This approval and acceptance is contingent on the candidate's adequate performance and defense of this dissertation at the final oral examination. The inclusion of this sheet bound into the library copy of the dissertation is evidence of satisfactory performance at the final examination.
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CHAPTER I

INTRODUCTION

A. DISCUSSION OF ST. LOUIS ENCEPHALITIS VIRUS

1. Strains of The Virus

St. Louis encephalitis virus was first isolated by Muckenfuss et al. (1933) from monkeys which had been inoculated with infected human brain. Syvertson and Berry (1935) used the "Daily" strain obtained from Dr. Muckenfuss. Webster and Fite (1933) independently recovered the virus using white mice.

Sabin and Buescher (1950) reported that while no hemagglutinin was demonstrable in the "Webster No. 3" strain which had had many passages in mice over a period of 17 years, hemagglutinin could be demonstrated with three different recently isolated strains, namely "Winkler" (3 to 6 mouse passages), "Parton" (passage 2 and 3) strains derived from patients with encephalitis and "MP 126" representing the second mouse passage from pools of Culex tarsalis mosquitoes. Strain "MP 388" which was recovered from Culex stigmatosoma in embryonated chicken eggs and "MP 395" which was recovered from Culex stigmatosoma by mouse inoculation as well as the Winkler and the Parton strain were studied for their ability to yield stable hemagglutinin by Chanock and Sabin (1953).

In a comparison of the immunological and biological properties
of several strains of St. Louis virus, Sather et al. (1953) report using "Webster No. 2", "Webster B-33", Winkler, "BFS 2132" (obtained from California mites), and in some of the tests, the "Hubbard" strain. The Hubbard strain was used in a study by Sulkin et al. (1960) and Kissling (1957) used both the Hubbard strain in 103rd mouse passage (Hubbard M₁₀₃) and strain "904" in 3rd mouse passage (M₃).

The prototype virus "TR 9464" used by the Trinidad Regional Virus Laboratory of the Rockefeller Foundation was isolated from mosquitoes Psorophora ferox in 1955 and is closely related to the Parton strain of St. Louis virus, Downs, 1958). Ten additional strains were isolated during the period from 1953-58; one from human serum, 4 from bird sera and 5 more from Culex species of mosquitoes. All are designated as "TR" strains. Human serum survey data from the report quoted above indicate that St. Louis immune rates were higher in Jamaica than those encountered anywhere else in the Caribbean.

A great number of strains have been isolated from human, bird, mosquito and mammal sources at the Viral and Rickettsial Disease Laboratories of the California State Department of Health, 87 in 1954, 14 in 1955, 2 in 1956, and 72 in 1957. (Johnson 1957) Lennette (1946) has described some characteristics of the Winkler strain isolated in 1945 by this laboratory. Sharples et al. (1950) report the use of the 'Brown' strain of St. Louis encephalitis virus.

In February 1960, the Virus Section of the Department of Bacteriology, University of Arizona received the Winkler and "Ruis" strains of St. Louis encephalitis virus from the Viral and Rickettsial Disease Laboratories of the California State Department of Health at Berkeley.
through the courtesy of Dr. Edwin Lennette. Both of these strains are of human origin.

In summary, there are innumerable strains of St. Louis encephalitis available with varying biological and immunological properties. There is however, no evidence to suggest subgroups within those viruses identified as St. Louis encephalitis virus. Since the method of choice for isolation and passage is intracerebral inoculation of mice, preferably suckling mice for isolation, and 3-4 weeks of age for passage, the virus is sent or transported in the form of whole mouse brain or 20% suspension in a suitable diluent in sealed ampules or tubes under dry ice.

2. Relationship of St. Louis Encephalitis Virus to Other Arthropod-borne Viruses

Although there is no evidence of subgroups in viruses identified as St. Louis encephalitis virus, there is a great deal of evidence to indicate an overlapping of biological, immunological and other properties of these viruses with various arthropod-vectored (Arbor) viruses. Smith and Lennette (1939) found that Japanese B encephalitis (JBE) virus and St. Louis (SLE) virus produce the same type of changes in the chorioallantoic membrane and the brain of the chick embryo. The two viruses multiplied in the egg to approximately the same titer as demonstrated by mouse inoculation. Macroscopic and microscopic examination of the infected chorioallantoic membrane yielded the same pattern of response for both. Macroscopically at 3-4 days after inoculation of the chorioallantoic membrane with 0.05 ml. of supernatant from a 10% broth suspension of mouse brain, there was conspicuous edema of the membrane with
slight opacity and very fine stippling. Microscopically, there was
diffuse proliferation of ectoderm with focal accentuation. Downgrowth
of the ectoderm into the mesoderm also occurred. Mesoderm was edema-
tous and there was moderate proliferation of fixed mesodermal cells and
some infiltration of wandering cells. No specific cellular inclusions
were seen.

Over a number of years information has accumulated indicating
that by neutralization, complement fixation and cross resistance tests,
certain immunological relationships could be established among certain
viruses of the arbor group. Smithburn (1942) showed a relationship to
exist between Japanese B, St. Louis and West Nile (WN) viruses. Casals
and Webster (1944) showed that louping-ill and Russian Far Eastern
viruses were closely connected, almost identical agents. Sabin (1952)
found by complement fixation that the viruses of dengue type 1, dengue
type 2, Japanese B, West Nile and Yellow fever had common antigenic con-
stituents. Havens et al. (1943) using complement fixation tests, re-
ported the crossing of Eastern (EEE) and Western equine encephalitis
(WEE) viruses but no detectable relationship between SLE, JBE and WN.
Improved techniques resulted in uncovering serological crossings. Sabin
emphasized the usefulness of high titered antigens in his work on com-
plement fixation and in his laboratory and that of the Rockefeller In-
stitute for Medical Research, the development of highly potent comple-
ment-fixing antigens extracted with acetone-ether led to the discovery
of additional crossings among arthropod-borne viruses which form the
basis for the arbor groupings.

The presently accepted classification of arthropod-borne viruses
is based on serologic cross reaction between viruses. (Casals, 1957)

According to Casals and Reeves (1959) three different tests, complement fixation (CF) hemagglutination inhibition (HI) and neutralization (NT) have been used for the detection of cross reactions upon which the present classification of the arbor viruses is based. All cross reacting viruses have been placed in a group and no virus has been found so far to react with more than one group. Sera produced by repeated injections of a virus into an animal generally have shown a broader range of overlap within the group than sera produced by a single injection. There are three groups of arbor viruses recognized: A, B, and C. The HI test is the basis for this grouping, for it has given a broader spectrum of cross reactions than the CF test, while the intracerebral NT has been the most specific. Viruses that have cross-reacted in CF and NT tests have always done so within the group as established by HI tests. In a more recent paper Casals and Whitman (1960) say

"In the past, new antigenic groups of the arbor viruses have been designated alphabetically, however this type of nomenclature may with increasing numbers of groups, cease to be practical and furthermore, may result in conflicting designation by independent investigators. For these reasons, it is considered advisable at least pending final solution of the problem of nomenclature of the arthropod-borne virus groups, to designate this new group by the name of one of its members. Accordingly the designation Bunyamwera group has been selected which includes Bunyamwera, Wyeomyia, Cache Valley, Kairi and Germiston viruses."

Within groups A and B, there are subgroups of viruses that are more closely related to each other than to other viruses in the group. These are Group A subgroup (1) Chikungunya, Mayaro, Semiliki Forest, subgroup (2) Sindbis, Western Equine (WEE), Group B subgroup (1) Dengue
types 1 and 2 subgroup (2) Ilheus, Japanese B (JBE), Murray Valley Encephalitis (MVE), St. Louis Encephalitis (SLE), West Nile (WN). Subgroup (3) Diphasic meningoencephalitis, Kyanasur Forest Disease (KFD), Louping-ill, Omsk hemorrhagic fever, Russian spring-summer encephalitis (RSSE). Subgroup (4) Uganda S, yellow fever (YF) and possibly Zika.

Subgroup 3 is composed of agents so closely related serologically, that it is questionable whether they are different entities.

It is to be emphasized that the Casals' classification omits all other biological properties of the viruses in question such as host range, vector, virulence by different routes of inoculation, length of incubation and type of disease produced in man and laboratory animals.

St. Louis encephalitis virus (SLE) is a group B arbor virus and a member of the subgroup which includes Ilheus, JBE, MVE and WN. The following table is taken from Olitsky and Clarke (1959).

**Group B, Arbor Viruses**

<table>
<thead>
<tr>
<th>Subgroup 1</th>
<th>Subgroup 2</th>
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<tbody>
<tr>
<td>Dengue 1</td>
<td>St. Louis</td>
</tr>
<tr>
<td>Dengue 2</td>
<td>Japanese B</td>
</tr>
<tr>
<td></td>
<td>Murray Valley</td>
</tr>
<tr>
<td></td>
<td>West Nile</td>
</tr>
<tr>
<td></td>
<td>Ilheus</td>
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<thead>
<tr>
<th>Subgroup 3</th>
<th>Subgroup 4</th>
</tr>
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<tbody>
<tr>
<td>Russian Tick-Borne Complex</td>
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<tr>
<td>Russian-Spring-Summer</td>
<td></td>
</tr>
<tr>
<td>Louping-Ill</td>
<td></td>
</tr>
<tr>
<td>Central European Tick-Borne</td>
<td></td>
</tr>
<tr>
<td>Biundulant Meningoencephalitis</td>
<td></td>
</tr>
<tr>
<td>(Diphasic Milk Fever)</td>
<td></td>
</tr>
<tr>
<td>Omsk Hemorrhagic Fever</td>
<td></td>
</tr>
<tr>
<td>Kyanasur Forest Disease</td>
<td></td>
</tr>
</tbody>
</table>

|                     | Yellow Fever |
|                     | Uganda S     |
|                     | Zika (possibly) |

The following members of Group B are not considered at present
as members of any particular subgroup: Ntaya, Spondweni, Wesselsbron, and Bat salivary virus. The possession of common antigenic constituents among the members of the same group of arbor viruses seems to reflect deeper relationships. It has been demonstrated in experimental animals that infection with one virus of a group will confer increased resistance to a second virus of the group and that mutual interactions between two viruses of a group result in serological response beyond the ability of each one separately. Hammon et al. (1956) suggest that immunity acquired by active immunization with one member of the group may serve as a good basic or primary immunization for other representatives and that a high and probably protective response can be stimulated subsequently by giving one "booster" injection of a killed virus vaccine representing an immunologically related virus. Complete protection of hamsters against WN and MVE viruses was afforded by JBE immunization and protection of a lesser degree obtained by SLE immunization. (Hammon and Sather, 1956) Price (1957) has presented evidence for the possibility of using in humans, a live WN virus in combination with one injection of killed JBE virus to protect against several related virulent viruses of the group B classification. Antibodies against JBE were demonstrated in terminal cancer patients deliberately inoculated with WN (Egypt 101 strain) and Ilheus viruses by Southam and Moore (1954b).

Sabin (1959) has written

"In my opinion, the assumption that infection with any group B virus produces a significant durable immunity to infection with any other member of the group is not supported by available data. Homotypic immunity is of long duration, while heterotypic falls with time. It has occurred to several investigators that a preceding infection with one group B virus might greatly improve the immunogenic response to a killed virus vaccine against another. Here
also, it has been found that the closeness of the antigenic relationship is of importance. Thus, while neither dengue nor 17D yellow fever virus infection altered the response of human beings to a single dose of killed JBE vaccine, a preceding infection with WN resulted in a prompt and significant antibody response not only to the JBE but also to the MVE and SLE virusus. The antibodies induced in this manner did not persist very long, and the practical significance of such maneuvers cannot be evaluated until much more work has been done in this field."

St. Louis encephalitis virus is further characterized by its diameter of 20-30 mu as determined by gradocol membrane filtration. It is pH and temperature sensitive. Duffy (1946) in a study of pH stability at a storage temperature of 4°C, found that the virus of St. Louis encephalitis could still be detected by infectivity for mice, after three weeks at pH 8.4 and pH 8.8 but not at higher or lower pH values. According to Chanock and Sabin (1953a) optimal pH conditions for stability of infectivity and hemagglutination ability diverge, that of hemagglutination (HA) was preserved best in the alkaline range (pH 9.0), while infectivity was retained at or slightly above neutrality (pH 7.0). Sulkin and Zarafonetis (1947) found that SLE as well as WEE and EEE were susceptible in vitro to concentrations of ethyl ether 50 to 100 times the maximum tolerated by their experimental animals, the average LD$_{50}$ being reduced more than 5 log units. Polio and rabies viruses were refractory to the same amounts of ether in vitro.

Theiler (1957) reported that SLE as well as all arbor viruses tested were inactivated by a 1:1000 solution of sodium desoxycholate. In the case of SLE between 3.5 and 4.1 logs of virus were inactivated. Polio, mouse encephalomyelitis, Coxsackie and encephalomyocarditis viruses were resistant to the action of sodium desoxycholate.

Cheng (1958) found that trypsin, chymotrypsin and papain
inactivate both the hemagglutinin and the infectivity of Group B arbor viruses (including SLE), but not of Group A viruses and cites evidence for inactivation as a result of direct proteolytic action on the virus particles.

A most interesting biological property and one of some practical importance shared by SLE and members of its subgroup i.e., MVE, WN, JBE, Ilheus, is an oncolytic effect. Sharples et al. (1950) reported that a transplantable lymphoid tumor of chickens can be caused to regress without any apparent damage to the host by superimposed inoculations of SLE, WN, JBE, Louping-Ill or Russian Spring-Summer encephalitis (RSSE) viruses. Chickens which have been inoculated with any of the five viral agents are rendered immune to challenge by homologous tumor even though the original inoculum with tumor may not have produced a palpable tumor. Pollard and Bussell (1952) reported that tumor implants growing in roller tube cultures demonstrated a marked oncolytic response to SLE infection. Two human and two rodent tumors were completely destroyed by SLE virus, less completely by neurotropic influenza virus. These authors stated in a note that in vivo studies have indicated that RSSE, SLE and Bunyamwera virus would parasitize and destroy transplantable mouse sarcomas and also that Egypt 101 virus (West Nile) destroyed human epidermoid carcinoma growing in X-irradiated rats and in tissue culture.

This oncolytic property of various members of the group B arbor viruses has provided research impetus both toward elucidation of their mechanism of action on cells as exemplified by the studies of Love (1959) and toward their use as anti-cancer agents. West Nile viruses, especially Egypt 101 strain, have received attention as anti-neoplastic agents since
it has been shown by Southam and Moore (1951) that West Nile virus inoculated into patients with advanced inoperable neoplastic disease produced an asymptomatic infection.

In a more detailed study of 90 patients with advanced neoplastic disease inoculated with Egypt 101 strain of West Nile virus, Southam and Moore (1954a) reported that patients with neoplastic disease of reticulo-endothelial cell origin had a striking predisposition to prolonged viremia and increased severity of clinical illness. The distribution of virus in tissues obtained at autopsy was studied in 15 patients who died within 4 weeks after inoculation of virus. Virus was demonstrated in neoplastic tissue of various histopathologic types. The normal tissues most frequently containing virus were spleen, lymph nodes, liver and lungs, suggesting distribution determined by reticulo-endothelial cells. Cytological studies on Ehrlich Ascites tumors infected with West Nile virus indicate infection produces abnormal nuclear changes ending in karyorrhexis. Preceding destruction, striking changes in the amount and distribution of neutral lipides, the lipochondria and mitochondria as well as impairment of cytochrome oxidase and succinic dehydrogenase activity are described by Orsi et al. (1957). An increase in the number and size of lipochondria without apparent change in mitochondria in RPL-12 Chicken Lymphoma cells infected with the NFT strain of St. Louis encephalitis virus has also been reported by Love and Sharples (1954).

That phospholipids might be important in connection with SLE and other viruses is indicated by two recent papers. Sulkin et al. (1960) using the Hubbard strain of SLE, inoculated subcutaneously into Mexican
free-tailed bats, were able to demonstrate virus in the brown fat and not in the blood in 3 of 7 instances and indicate that there is a demonstrable affinity of SLE and JBE viruses for brown fat tissue. Porterfield and Rowe (1960) report inhibition of group B hemagglutinin activity by choline containing phospholipids related to lecithin. In summary, St. Louis encephalitis virus shares antigenic constituents with the group B arbor viruses and is sensitive to pH and temperature. It is a member of the West Nile subgroup which includes in addition to West Nile (WN) and St. Louis encephalitis (SLE), the viruses of Japanese B encephalitis (JBE), Ilheus, and Murray Valley encephalitis (MVE). It shares with these viruses in varying degree the following properties: immunological overlap; inactivation by ether, sodium desoxycholate, trypsin, chymotrypsin and papain; hemagglutination which can be inhibited by phospholipid; lipotropism; oncolysis for certain tumor tissues.

3. **Cultivation of St. Louis Encephalitis Virus**

Although St. Louis encephalitis virus was originally isolated by passage of human brain material into monkeys and then carried in mice which are at present the animals of choice for isolation attempts, (Hammon, 1956), early attempts at cultivation of the virus in vitro with a modified Maitland technique were quite successful, (Syverton and Berry, 1935), (Harrison and Moore, 1936), (Schultz, Williams and Netherington, 1938), (Malloy, 1940). Infectivity of the serially passaged virus was demonstrated by intracerebral inoculation of 3-4 week old white mice.

Harrison and Moore (1936) demonstrated the propagation of the virus of St. Louis encephalitis in embryonated eggs, and Schultz et al. (1938) serially passaged the virus on the chorioallantoic membrane (CAM)
of embryonated eggs as well as with the Maitland type technique using minced mouse embryo as the tissue component. In all of the Maitland type techniques, although it was possible to serially propagate the virus, the titers of the infective fluids were uniformly low (approximately $10^{-2}$). Malloy (1940) obtained higher titers in a medium containing embryonic mouse or guinea pig brain in ox serum ultrafiltrate when incubated at room temperature rather than at 37°C. Infected chorioallantoic membranes maintained in serum ultrafiltrate at room temperature supported growth of St. Louis virus to titers of from $10^{-4}$ to $10^{-5}$. In view of the extreme temperature sensitivity of SLE, it seems possible that the higher titers might have been due to less rapid loss of infectivity in the virus producing system rather than to media components or other factors.

Although Pollard and Bussell (1952) cultivated SLE in tumor explants growing in roller tubes in order to study its oncolytic effect and Pollard (1955) reported on the effect of SLE infection of mouse spleen explants, it is rather striking that so few studies of SLE in trypsinized monolayer tissue cultures (or cell cultures as proposed by Ross and Syverton) has been attempted. There are numerous reports of such studies using viruses of the arbor B subgroup 2 to which SLE belongs. (Noyes, 1955), (Bhatt and Work, 1957), (Banta, 1958), (McCollum and Foley, 1957), (Diercks and Hammon, 1959), (Porterfield, 1960a), (Buckley, 1959). St. Louis encephalitis virus is conspicuous by its absence.

There have been a few reports of studies including St. Louis encephalitis virus in trypsin dispersed cell monolayers. Scherer and
Syverton (1954) using the Hubbard strain of SLE indicated that cytopathogenic effects of SLE virus for strain HeLa cell were observed infrequently and irregularly, yet the virus multiplied. Nine serial passages of virus extending over a 72 day period were carried out successfully. The pooled cultural fluids from each passage produced in mice signs of encephalitis and death although a cytopathogenic effect with destruction of cells was observed only in passages 1, 6, and 7.

Kissling (1957) reported cytopathogenesis in hamster kidney monolayers infected with EEE, WEE, VEE, SLE, JBE, MVE, WN, and Ilheus viruses. Guinea pig kidney and chick embryo cultures reacted with complete cytopathogenesis only with EEE, WEE and VEE viruses.

Diercks and Hammon (1958) report that in screening tests with 13 arbor agents and 3 types of polio viruses, hamster kidney monolayer cell cultures proved markedly susceptible to the cytopathogenic effect (CPE) of JBE, WN, Uganda S, Ilheus, Bunyamwera, and Semiliki Forest Viruses, only moderately susceptible to the effects of SLE and the New Guinea C Hawaiian and Trinidad strains of dengue virus. The Ntaya, Zika and Bwamba agents and the 3 types of poliovirus failed to show a cytopathogenic effect.

Lenahan and Wenner (1960) in a study of ECHO, Coxsackie, Simian, Adeno, Arbor and other viruses in a variety of renal cells obtained from non-primate hosts reported that their strain "B33" of St. Louis encephalitis virus produced slow (5-8 days) cytopathogenic action in hamster kidney cell monolayers but the virus levels were low on titration in homologous cultures. Under the conditions of their tests, cat kidney cells responded in a manner comparable to hamster kidney cells, while
pig kidney cells responded with more rapid action (2-4 days).

Henderson and Taylor (1959) in a study of arbor viruses demonstrated plaque production by SLE in Peking duck kidney cell monolayers overlaid with agar containing neutral red. Strain "RF B-50487" which had had 122 mouse passages gave comparable titers by infant mouse titration and plaque production (5.1 and 5.8 logs respectively), but titration by cytopathogenic effect in the cell monolayers titered less than 2.0 log units.

In summary,

St. Louis encephalitis virus has been cultivated in Maitland type tissue cultures and in embryonated eggs and although serial passage was possible, high yields of virus were not obtained. Growth in various trypsinized cell monolayers has been reported but cytopathogenesis is irregular. The conflicting reports of Kissling and Lenahan and Wenner as to infectivity of hamster kidney cell culture fluids for the homologous system was probably due to differences in laboratory procedure.
Mass produced cell cultures are so widely used in virology at present that they are considered a primary and indispensable tool. This has been made possible through the use of antibiotics to control contamination and trypsin to disperse cells. Trypsinization of tissues was originated by Rous and Jones (1916) and reintroduced by Scherer, Syverton and Gey (1953). Replicate primary cell cultures of certain human and animal tissues both embryonic and adult and a variety of cell strains in continuous culture (mostly neoplastic) have been used for a wide variety of problems.

Enders et al. (1949) demonstrated that poliomyelitis virus, a typical neurotropic virus could be propagated in tissues of non-nervous origin. This work gave tremendous impetus to the field of virology. The voluminous literature that has accumulated since on poliovirus in tissue culture, leads the field by far on morphological and functional changes that occur in cells infected with viruses.

According to Syverton (1957) cellular change due to virus infection extends from simple alteration in physiological activity to total necrosis, from necrosis to hyperplasia and from hyperplasia to neoplasia. Cytopathic alterations are recognized as clustering about five different types. Total destruction is the usual result of infection by polio, Coxsackie B, a few Coxsackie A and some ECHO viruses. Subtotal cellular degeneration is produced by viruses of the arthropod-borne encephalitogenic group and by Newcastle disease virus. Viruses of the herpes B-pseudorabies group slowly produce a focal degeneration with
centrifugal progressive involvement. Adenoviruses produce cytopathic changes without cessation of cellular metabolism, accumulating fluid, distending and aggregating into refractive clumps. Measles virus and simian orphan viruses produce degeneration with affected cells coalescing into syncytial clumps sometimes including 40 or more nuclei.

It is most important to emphasize, however, that the effects described above are meaningful only within the framework of the tissue culture technique employed to obtain them. They are generalizations of results obtained primarily with monolayer cell cultures of stable cell lines such as HeLa, KB, Detroit-6, Maben, etc. The cell virus interactions in this closed system are such that they have extremely limited, if any, applicability to the pathogenesis of the virus in the intact animal. Even though varicella, herpes zoster and measles each show rather unique cytopathic changes in tissue culture and there appears to be some correlation between the changes produced and the lesions produced in the human host tissue, this is the exception rather than the rule. (Cheatham, 1959)

The unique contribution of the trypsinized monolayer techniques to virology is the expansion by 100 to 500 fold of biological material available for experimentation. For the purpose of viral assay, each tube takes the place of one animal. Because of its much greater simplicity as a biological system, the cell monolayer is much less susceptible to uncontrolled environmental variables. In other words, it is more amenable to controlled experimental conditions. "The fact that many species of mammalian viruses have been found to exhibit cytopathogenicity under appropriate conditions has rendered the tissue culture
independent of the experimental animal as a means of detecting the presence of cytopathogenic viruses and as a measure of their infectivity." (Enders, 1957)

For those virus-cell systems in which cytopathogenesis proceeds regularly, e.g., polio in monkey kidney or in HeLa cells, there is no problem in quantitating viral activity by means of cytopathogenesis. For those virus-cell systems in which cytopathogenesis is irregular and virus increase proceeds without accompanying cell destruction, quantitation of virus to a level of accuracy obtainable with polio virus is impossible. St. Louis encephalitis virus in various cell systems has been so reported. Therefore, it appeared that an inquiry into cytopathogenesis in virus cell systems and reports of alteration of cytopathogenesis might prove rewarding.
2. **Factors Which Influence Cytopathogenicity**

**Passage History of The Virus**

Whether one ascribes the underlying mechanism to adaptation or to selection, which is the more ascendant theory at present, it is axiomatic to virologists that the passage history of the virus influences its performance. It was by prolonged cultivation in tissue culture that the 17D strain of yellow fever virus was developed by Theiler and Smith (1937a,b) for use in human immunization.

Mayyasi _et al._ (1960) by passage of the virus of Venezuelan Equine encephalitis (VEE) through a line of cells derived from monkey brain, produced a variant which differed from the parent virus in being more virulent for monkey brain cells and less virulent for L cells. The greater virulence of the variant for monkey brain cells was manifested as follows: a) the cells could be infected with much lower doses of the variant, b) the variant produced a cytopathogenic effect earlier than the parent and it was more intense and c) growth of the variant virus in monkey brain cells was more rapid than the parent.

Kissling (1957) using hamster kidney cell cultures, obtained 1.5 log units lower cytopathogenic dose endpoint with the Hubbard strain of SLE which had had 103 mouse passages as compared to strain "904" of SLE which had had only 3.

The adaptation of two strains of WN virus, of Tamil Nad virus and of JBE to chick embryo cell and monkey kidney cell cultures is described by Bhatt and Work (1957). After a variable number of passages, cytopathogenicity was sufficiently enhanced that tube cultures could be used for virus titration with all three viruses.
Type of Cell.

While it is very difficult to classify tissue culture cells on the basis of morphology, it is possible in general to separate them into cells of epithelial type and cells of fibroblast type. Some viruses will attack both but others will preferentially attack the epithelial type of cell, leaving the fibroblast type almost untouched. This is particularly evident in the case of the adeno-viruses. (Paul, 1959) Some specificity in the infectivity of different types has also been shown in organ culture by Bang (1955). When small organized rudiments of tissue are infected with viruses in vitro, it appears that the lesions are frequently confined to one particular type of cell within the tissue.

It has been shown by Hsiung and Melnick (1955, 1957) that monkey kidney cell cultures derived from the capuchin monkey will not support growth of polio virus while those of rhesus and macaque monkeys are destroyed with great regularity. Bang and Gey (1952) published the results of a study on the varying susceptibility of thirteen established strains of rat cells to Eastern equine encephalomyelitis virus. Included among the cell strains were both malignant and normal lines. A broad spectrum of variation was demonstrated ranging from complete or almost complete resistance to extreme susceptibility characterized by rapid multiplication of virus and destruction of cells. Partially resistant lines continued to support virus growth for many months and through a series of transfers. In certain instances the virus eventually disappeared from cultures in which the cells continued to proliferate. Such variations were not dependent on the malignancy of the cell line.

With reference to SLE, it has been previously mentioned in the section on performance of SLE in tissue cultures that Kissling found
cytopathogenesis produced in hamster kidney cell cultures but not in
guinea pig kidney, dog kidney or in chick embryo cell cultures.
Lenahan and Wenner's (1960) studies, also mentioned previously, indicate
SLE produced cytopathogenic changes in hamster, cat and pig kidney cell
cultures, but not in monkey, calf, lamb, dog, rabbit or mouse kidney
nor in human amnion cell cultures.

Medium Factors

Tissue culture nutrition and metabolism if it is not already,
is certainly rapidly becoming a field in itself. The availability of
tissue culture media from an increasing number of commercial sources
emphasizes the trend toward standardization of media and techniques in
tissue culture virology which the work of Hanks, Gey, Earle, Morgan,
Morton and Parker, Melnick, Eagle and others helped accomplish.

Medium factors have been specifically implicated in cytopatho-
genesis in virus cell culture systems in several instances. Reissig
et al. (1956) have shown that with measles virus in a human epidermoid:
cell line Hep-2, two completely different morphological patterns of
degeneration could be obtained depending upon the composition of the
nutrient medium employed for virus propagation. Hep-2 cells grown in
Eagle's basal synthetic medium supplemented with 10% calf serum were
inoculated with the Edmonston strain of measles virus. At the time of
inoculation, as is usual, the growth medium was replaced. Either fresh
Eagle's medium containing 5 or 10% calf serum or a biological medium
recommended by Enders, composed of 35% bovine amniotic fluid, 5% beef
embryo extract, 25% heated horse serum and 35% Hanks' balanced salt
solution was used. Cytopathic changes appeared from 2 to 14 days after virus infection depending on the dose of virus inoculated. When Enders' medium was used as a nutrient, flat syngial masses appeared in the inoculated cultures, some of the giant cells containing several hundred nuclei. In the cultures nourished in Eagle's medium however, the cells after infection became either elongated and spindle-shaped or rounded with very few polynucleated cells. When Enders' medium was supplemented with the amino acids, vitamins and glutamine contained in Eagle's medium, or with glutamine alone, giant cell formation was greatly reduced and most of the cells involved were typical of measles degeneration as in Eagle's medium alone. That the effect could be attributed primarily to glutamine was proved by its omission from the Eagle's medium. With the glutamine deficient medium, the appearance of infected cultures was very similar to that in Enders' medium.

Differences in plaque production by attenuated and virulent strains of polio viruses have been described by Melnick (1957) as due to the bicarbonate concentration of the medium.

Up to 89% reduction in plaques produced by WEE in chick embryo cell monolayers has been shown to be due specifically to neutral red in the agar overlay medium by Darnell, et al. (1958). The inhibitory effect of neutral red on plaque formation by foot and mouth disease virus type A in primary calf kidney tissue cultures has also been reported. Pledger, 1960), McClain and Hackett (1958) reported as high as 73% reduction of plaques formed by vesicular stomatitis virus (VSV) in chick fibroblast monolayers by neutral red.
3. Cellular Immunity Mechanisms

Specific cytopathogenesis of a virus in a tissue culture system can be modified by serum containing specific viral antibodies and serves as the basis for the tissue culture neutralization test which is widely used for identification of viral isolates, diagnosis of viral infections, etc. Successful results in such tests depend upon mixing the immune serum with the virus inoculum before addition to the tissue culture system, for it is generally conceded that once adsorption of the virus to susceptible cells has occurred, immune serum has no protective effect in preventing cytopathogenesis.

It is the specific immunity of cells to viruses in the absence of humoral antibodies that can be studied profitably by tissue culture techniques. Such cellular immunity could be based on lack of suitable cell receptors so that virus adsorption is prevented, capacity of cells to harbor latent virus analogous to temperate bacteriophage with consequent immunity to super-infection, failure of the cells to reproduce adsorbed virus in infectious form, or mechanisms involved in viral interference.

That such studies are of great importance in delineating concepts of viral immunity as well as immunity in general is implied in an article on a new approach to immunology by Burnet (1961).

"It is far from certain that selection theories of antibody production and immunity will prove correct. The clonal selection theory has already had to be modified importantly to remain in line with the facts. Like every other scientific hypothesis, it will have to go on fighting a never-ending rear-guard action until eventually it becomes unrecognizable. Current ideas and controversies, however, have completely validated a point of view that was only beginning to emerge when they were initiated. This is that the processes of immunity are essentially a matter of the
population dynamics of mesenchymal cells within the body. Antibody production is something of only secondary importance. This change of values will persist. It will undoubtedly alter the line of experimental approach, and it may be hoped that it will play an important part in the elucidation of one of the most urgent of the problems of medical science...autoimmune disease."

In studies of mammalian cell virus relationships, McClaren et al. (1959a) demonstrated that primary or established strain cultures of a variety of primate cells that are susceptible to cytopathic infection with polio virus strongly adsorbed the virus while insusceptible non-primate cells in primary or established-strain culture did not. In a second paper these same authors (1959b) indicate that their findings suggest that the capacity to adsorb, receive and eclipse polio-virus is associated with organized cytoplasmic lipo-protein structures not possessed by insusceptible cells.

Lockhart (1960) has demonstrated that L cells (mouse fibroblast-malignant) exposed to large amounts of heat-inactivated WEE virus, become refractory to destruction by active WEE virus and such cells neither yield virus nor cytopathic effects. Cells which have so been made resistant still adsorb active virus. The resistance is retained through several but not an indefinite number of cell divisions in the absence of any further additions of virus. Cultures of cells which have been rendered resistant to infection by treatment with inactive virus may readily become persistently infected with active WEE and the persistence of this chronically infected state may be due to the continued operation of the interference mechanism.

Both homologous and heterologous interference may be mediated through interferon, an antiviral substance produced in chick embryo fibroblast, calf and rabbit kidney cells and human cell lines. (Isaacs
Interferon was first demonstrated by Isaacs and Lindemann (1957) during studies of interference produced by heat inactivated influenza virus in fragments of chick chorioallantoic membrane. It was observed that when the inactive virus and tissue were incubated together, a virus-interfering substance was produced with physico-chemical properties different from those of inactive virus.

Among the interesting properties of interferon are that it is a protein of low toxicity to tissues and that it is apparently non-antigenic and able to inhibit the growth of a large variety of viruses. Interferon prepared by incubating chick embryo cells with inactivated influenza virus can inhibit the growth in tissue culture of the small sized encephalitis viruses, medium-sized viruses such as the influenza viruses, and large viruses, like those of the pox group. (Isaacs and Westwood, 1959)

Porterfield (1959c) has demonstrated that interferon produces a zone of inhibition of virus multiplication in sheets of cells infected with plaque producing viruses. Since the area of the zone of inhibition is directly proportional to the concentration of interferon applied, he suggests the technique may be used for assay of interferon with Bunyamwera virus as the plaque production agent.

A viral inhibitory fluid produced in primary human kidney and amnion cell cultures infected with a chick embryo adapted strain of type 2 poliovirus (RMC) has been described and contrasted with interferon by Ho and Enders (1959). These authors indicate that viral inhibitory fluid (VIF) produced by cells might possibly be involved in the well recognized variation in susceptibility of different cells and organs or in the length
of the incubation period. They contend that study of inhibitory sub-
stances of this class may contribute substantially to the understanding
of mechanisms of virus infection and disease.

That the cytopathogenic effect of various viruses in tissue culture
can be modified by anticellular serum has been demonstrated by Quersin-
Thiry (1958). Heated rabbit anticellular sera used at appropriate con-
centrations to dilute out the agglutinative effect can immunologically
block the cellular cultures. Virus infection is inhibited although the
cells remain alive. The efficiency of protection is high against some
viruses, e.g. ECHO type 9, moderate against some e.g. polioviruses and
weak or nil against several types e.g. Coxsackie B and Newcastle Disease
virus (NDV). Similar results have also been published by Habel et al.
(1958).

In further work with rabbit anticellular sera prepared against
twenty three different stable cell lines and five types of trypsinized
primocultures, Quersin-Thiry (1959) has indicated that during the first
week of cultivation, trypsinized monkey kidney (MK) cultures seem to
acquire a new antigen which is present in cultures of the HeLa group but
absent in the non-cultivated monkey kidney organ. Homografts of monkey
kidney will support multiplication of poliovirus and anti-sera prepared
against these grafts exhibited a strongly protective effect on MK cultures
infected with virus. Antisera prepared against kidney suspension (not
cultivated) were only weakly active. It is possible that homografts have
an ability like that of MK cultures in continuous cultivation in vitro,
to elaborate the antigen linked to virus susceptibility. In comparing
the agglutinative effect and protective effect against poliovirus of the
anticellular sera, the following conclusions were reached. Tests indi-
cated that HeLa cells and all other human cell lines susceptible to
polio virus as well as Salk's monkey heart cell line, belong to one homo-
genous antigenic group and that cross reactions between these lines are
complete. It is assumed that these lines share two antigens in common:
an antigen linked to susceptibility to polio virus and an antigen present
in human and monkey cells. As evidenced by partial or weak cross-reactions,
either of these two antigens may exist separately in some stable lines or
primocultures, e.g. the antigen linked to polio virus susceptibility in
Westwood's rabbit lines; a human antigen, very similar to a monkey
antigen in human and monkey cell lines which are not susceptible to polio-
virus. Cultures not of human or monkey origin and not susceptible to
polio virus had no antigenic relationship to human cell lines or to monkey
kidney primocultures (with one exception).

In contrast to those human cell lines which are susceptible to
polio virus (prototype HeLa), the human cell lines which are not virus
susceptible do not form a homogenous group. They do share some human
antigen in common but seem to contain another distinct antigen specific
for each line.

It seems possible that there is a relationship if not identity
between Quersin-Thiry's cell antigen linked to susceptibility to polio
and McLaren's et al. (1959b) organized cytoplasmic lipoprotein structures.

In earlier work on the effect of various anti-tissue sera prepared
in rabbits (anti-CAM, anti-mouse brain, etc.) against various viruses
(influenza, polio, LCM, WEE, etc.), the protective action of such sera is
described as due to inhibition of virus fixation and inhibition of virus
liberation. Cell receptors of virus are blocked by antibody. (Quersin-Thiry, 1955)

No comparable work has been done using St. Louis encephalitis virus in tissue culture. Pollard (1955) found that explant cultures of spleens taken from mice immunized with SL2 or mouse encephalomyocarditis (EMC) viruses were selectively immune to homologous virus and susceptible to heterologous virus but it is highly probable his results were due to antibody production in vitro rather than cellular immunity mechanisms.

IN SUMMARY: Cytopathogenesis produced by viruses in cell cultures, is dependent on many factors. Some of these factors which have been shown to modify the cytopathogenic effect are: the passage history of the virus, type of cell, inorganic and organic chemical components of the medium, specific antiviral antibody and cellular immunity mechanisms.
CHAPTER II

STATEMENT OF PROBLEM

St. Louis encephalitis virus is multiplied in the chick embryo and various tissue cultures, but the production of cytopathogenic effects is absent or irregular in many systems. Cytopathogenesis is now widely used for sensitive viral assay and there is evidence of a possible role of immunity at the cellular level in the mechanism of cytopathogenesis.

Because of Kissling's (1957) report on the cytopathogenicity of SLE in primary hamster kidney cell cultures, it was decided to use this cell system. It was thought desirable to compare the growth and cytopathogenic effect (CPE) of SLE in hamster kidney cell cultures (epithelial type) with that in another cell system taken from the same animal, preferably fibroblast type. The spleen was chosen because it is an easily accessible organ, rich in connective tissue and reticuloendothelial cells. Pollard (1955) in his paper on growth of SLE in mouse spleen explants, described the outgrowth as fibroblastic.

The problem as originally delineated therefore, was to be a study of the growth of SLE and its CPE in hamster kidney and spleen primary cell cultures from normal and immunized animals.

Results of attempts to obtain spleen cell cultures by trypsinization and/or collagenase treatment (Hinz and Syverton, 1959) are presented in section A.

The cytopathogenic effect (CPE) of the Winkler strain of St. Louis
encephalitis virus (SLE) in hamster kidney cell cultures and the effect of normal and "immune" rabbit serum outgrowth medium on the CPE of SLE in normal and "immune" hamster kidney cell cultures are presented in sections B and C.

Studies of the Winkler strain of SLE in chick embryo cell cultures are presented in section D.
CHAPTER III

MATERIALS AND METHODS

A. PREPARATION OF STOCK SOLUTIONS, REAGENTS, MEDIA AND SERA

In general, standard operating procedure used at the California State Department of Public Health Viral and Rickettsial Disease Laboratory, Berkeley, formed the framework for techniques, media and reagents used.

Balanced salt solutions, buffers, 1% trypsin solution for use in cell dispersal, antibiotic solutions, media, nutrient solutions and sera were all prepared as stock solutions. Most were filter sterilized under 5-8 lbs. positive pressure with a 100 or 250 ml. capacity Horm\(^1\) filter (glass, stainless steel seitz type) with a D-7 pad, (retains 0.1 u) pre-rinsed with phosphate buffered saline. In a few instances, e.g. bicarbonate solutions, lactalbumin hydrolysate, solutions were autoclaved at 10 lbs. (234°F.) for 10 minutes. During the filtration procedure solutions were dispensed into specially washed, rinsed and presterilized tubes or bottles in appropriate amounts. Each tube or bottle aliquot was tested for sterility in malt extract, yeast extract broth (MY).

\(^{1}\)F. R. Hormann and Co., Inc., 17 Stone St., Newark, New Jersey.
(Wickerham, 1951) for yeasts and molds, and trypticase soy semi-solid medium\(^2\) for bacteria. Sterility test media were routinely incubated at 37\(^\circ\)C. for 48 hours at least.

Sterile stock preparations were stored under appropriate temperature conditions (room, refrigerator or freezer) and when needed, measured aseptically in specially washed and rinsed sterile glassware. Dilutions were made with double distilled water, (dd\(H_2O\)) prepared by redistilling distilled water in an all glass still. Approximately 700 ml. amounts were dispensed to specially washed and rinsed liter erlenmeyers with white rubber lined screw caps, autoclaved at 121\(^\circ\)C. for 20 minutes and stored at room temperature.

For closure of all tissue culture tubes, bottles or flasks, either screw caps lined with white rubber or white nontoxic rubber stoppers\(^3\) were used. CP grade chemicals were used throughout.

\(^2\)Baltimore Biological Co., Trypticase Soy Broth with 0.1% agar added.

\(^3\)Obtained from West Co., Phoenixville, Pennsylvania.
1. **Hanks’ balanced salt solution (BSS) 10X concentration, 500 ml. was prepared as follows:**

Solution 1 (250 ml.)

<table>
<thead>
<tr>
<th>Salt</th>
<th>gms</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>40.0</td>
</tr>
<tr>
<td>KCl</td>
<td>2.0</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.5</td>
</tr>
<tr>
<td>MgCl$_2$.6H$_2$O</td>
<td>0.5</td>
</tr>
<tr>
<td>CaCl$_2$.2H$_2$O</td>
<td>0.67</td>
</tr>
<tr>
<td>ddH$_2$O to 250 ml.</td>
<td></td>
</tr>
</tbody>
</table>

Solution 2 (250 ml.)

<table>
<thead>
<tr>
<th>Salt</th>
<th>gms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$HPO$_4$.12H$_2$</td>
<td>0.76</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.3</td>
</tr>
<tr>
<td>Dextrose (CP, BBL)</td>
<td>5.0</td>
</tr>
<tr>
<td>ddH$_2$O to 250 ml.</td>
<td></td>
</tr>
</tbody>
</table>

Each salt was weighed and dissolved in double distilled water (ddH$_2$O) in the order given in a 250 ml. volumetric flask. Each addition was completely dissolved before the next was added. The solution was then made up to the mark with ddH$_2$O.

Solution 1 was poured into a liter erlenmeyer and swirled while solution 2 was slowly poured into it. This prevented precipitates forming. Ten ml. of 1% Phenol red solution was added (see p. 33) to the final mixture and after thorough mixing it was filtered through a 250 ml. capacity Horm filter (D-7 pad) under 4-6 lbs. pressure, and dispensed in approximate 50 or 100 ml. amounts in prescription bottles. Each aliquot was tested in MY broth and trypticase soy semi-solid medium for sterility, labelled Hanks’ 10X, with the date of filtration and stored at room temperature.

As needed this 10X Hanks’ BSS was measured into a sterile graduate, diluted 1 to 10 with sterile ddH$_2$O and used as a component of media, for
rinsing tissue or cell suspensions, as a diluent for stock trypsin solution, etc.

2. Earle's balanced salt solution 10X concentration

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount (gms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>68.0</td>
</tr>
<tr>
<td>KCl</td>
<td>4.0</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2.0</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>1.4</td>
</tr>
<tr>
<td>NaH₂PO₄·1H₂O</td>
<td>1.4</td>
</tr>
<tr>
<td>Dextrose, CP</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Each reagent was dissolved completely in ddH₂O in the order given before the next was added. Sixteen ml. of 1% phenol red solution was added per liter and the solution filtered, dispensed, sterility tested and stored as with Hanks' B33.

As needed this 10X Earle's was diluted 1:10 aseptically with sterile ddH₂O and used for 1:10,000 neutral red solution or diluted appropriately for use in medium A.

3. Phenol red solution

100 ml. of 1% phenol red solution was prepared as follows:

a. Roughly 12 ml. of saturated NaOH was poured into a 15 ml. centrifuge tube and centrifuged at approximately 1800 rpm for 10 minutes to spin down carbonates. Five ml. of clear NaOH was then pipetted into a 50 ml. erlenmeyer and 45 ml. ddH₂O was added. This resulted in an approximately 1 N NaOH solution.

b. One gram of National Aniline phenol red (Phenolsulfonphthalein) NOT WATER SOLUBLE, was weighed into a 100 ml. volumetric. The 1 N NaOH (above) was added in small aliquots until the Phenol Red powder was
completely in solution (3-5 ml. were used). The solution was then
diluted to the mark with ddH$_2$O.

Excess NaOH produces a very dark purple solution which is not satisfac-
tory for use in tissue culture media. Phenol red solution is added in
appropriate amounts to balanced salt solutions and media as a pH indi-
cator. The final concentration is approximately 0.02%. Media pH was
thus adjusted colorimetrically under aseptic conditions by addition of
sterile buffer solution.

4. **Phosphate buffered saline (PBS) 10X concentration**

<table>
<thead>
<tr>
<th>Solution A (500 ml.)</th>
<th>gms</th>
<th>Solution B (500 ml.)</th>
<th>gms</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>40.0</td>
<td>Na$_2$HPO$_4$·12H$_2$O</td>
<td>14.5</td>
</tr>
<tr>
<td>KCl</td>
<td>1.0</td>
<td>KH$_2$PO$_4$</td>
<td>1.0</td>
</tr>
<tr>
<td>CaCl$_2$.2H$_2$O</td>
<td>.67</td>
<td>ddH$_2$O</td>
<td></td>
</tr>
<tr>
<td>MgCl$_2$.6H$_2$O</td>
<td>.5</td>
<td>to 500 ml.</td>
<td></td>
</tr>
</tbody>
</table>

These solutions A and B were stored at room temperature in separate 1
liter glass stoppered reagent bottles. To prepare 500 ml. of phosphate
buffered saline 1X for use, 50 ml. of solution A was added to 400 ml. of
ddH$_2$O in a liter erlenmeyer and then 50 ml. of solution B added to the
diluted A. This prevented precipitation of Ca and Mg salts. Phosphate
buffered saline (PBS)1X was used as a diluent for stock trypsin solution
and for rinsing Horm filter pads. Roughly 50 ml. was used and discarded
before addition of the solution to be filtered.
5. **Bicarbonate buffer solutions**

   a. 2.8 per cent sodium bicarbonate
   
   NaHCO₃ 2.8 gms.
   ddH₂O 100 ml.

   b. 8.8 per cent sodium bicarbonate
   
   NaHCO₃ 8.8 gms.
   ddH₂O 100 ml.

These solutions were dispensed into white rubber-lined screw cap test tubes in approximately 10 ml. amounts. Caps were tightened and tubes were autoclaved at 10 lbs. (234°F.) for 10 minutes. Tubes were stored at room temperature and used to adjust the final pH of solutions such as Hanks’, 0.25% trypsin, outgrowth or maintenance medium.

6. **Bovine albumin phosphate buffered saline solution (BABS)**

   a. 250 ml. of buffered saline was prepared as follows:

   NaCl 1.754 gms.
   Na₂HPO₄ (anhyd.) 0.632 "
   NAH₂PO₄·H₂O 0.076 "
   ddH₂O 250 ml.

   b. Bovine albumin powder (Fraction V)⁴ was dissolved in the above buffered saline solution to make a 0.75% solution e.g. 1.975 gms. The solution was sterilized by filtration through a 250 ml. Horm filter under 5 lbs. pressure (D-7 pad), dispensed directly into tubes, approximately 12 ml. per tube and each tube was tested for sterility in trypticase soy and MY broth. Storage of tubes was at refrigerator temperature (2-4°C.) BABS was used as a diluent for virus material in preparing dilutions for assay, 20% suspensions of mouse brain, etc.

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⁴Armour Labs., 168 2nd St., San Francisco, California, Lot #2293.
7. Medium A. \textbf{(Lactalbumin hydrolysate-yeast extract medium)}

For 1 liter 1X conc. \quad 1 liter 2X conc.

<table>
<thead>
<tr>
<th>Component</th>
<th>1X conc.</th>
<th>2X conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactalbumin hydrolysate (Difco)</td>
<td>5.0 gms.</td>
<td>10.0 gms.</td>
</tr>
<tr>
<td>Glucose (Dextrose, C.P.)</td>
<td>4.5 &quot;</td>
<td>9.0 &quot;</td>
</tr>
<tr>
<td>Yeast Extract (Difco)</td>
<td>1.0 &quot;</td>
<td>2.0 &quot;</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>1.1 &quot;</td>
<td>2.2 &quot;</td>
</tr>
<tr>
<td>10X Earle's BSS</td>
<td>100 ml.</td>
<td>200 ml.</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>900 ml.</td>
<td>800 ml.</td>
</tr>
</tbody>
</table>

Solids were best dissolved by allowing the mixture to stand overnight in the refrigerator. (4°C.) When solution was complete the flask contents was warmed to room temperature in a 37°C. water bath, filter sterilized, dispensed, sterility tested and stored to use as needed at 4°C. (refrigerator).

Medium A (1X) was used as the major component of tissue culture medium for guinea pig and hamster kidney cell culture work. Addition of antibiotic solution and serum results in the complete medium. Medium A (2X) was used to prepare double strength maintenance medium which when mixed with 4% agar in ddH$_2$O, served as the overlay medium in attempts at plaque production.

8. Eagle's L Medium

Eagle's L medium was purchased in 10X concentration from Difco. The addition of approximately 1 ml. of a 3-5% solution of glutamine per 100 ml. of medium and buffer (bicarbonate or tris hydroxymethyl aminomethane) for pH adjustment completed the medium for use in maintenance of chick embryo cell cultures.

9. Nutrient solutions

Twenty per cent glucose in ddH$_2$O, 5% yeast extract in ddH$_2$O and 5%
lactalbumin hydrolysate in 0.85% saline were prepared and either filter sterilized or sterilized by autoclaving at 10 lbs. (234°F.) for 10 minutes.

10. GNK is a solution of glucose, and sodium and potassium chlorides in concentrations appropriate to Hanks' BSS.

For 1 liter 1× concentration

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.0</td>
<td>gms.</td>
</tr>
<tr>
<td>KCl</td>
<td>0.4</td>
<td>&quot;</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.0</td>
<td>&quot;</td>
</tr>
<tr>
<td>1% phenol red</td>
<td>2 ml.</td>
<td></td>
</tr>
<tr>
<td>ddH₂O</td>
<td>to</td>
<td>1000 ml.</td>
</tr>
</tbody>
</table>

Each reagent was dissolved separately in the order given in ddH₂O in a volumetric. The solution was sterilized through a Horm D-7 pad dispensed, sterility tested and stored at room temperature.⁵ GNK solution was recommended as the diluent for collagenase for the collagenase dispersal of cells. (Hinz and Syverton, 1959) Collagenase (10 mgms)⁶ was dissolved in 100 ml. of GNK solution and sterilized by filtration through a 100 ml. Horm filter (D-7 pad) prerinsed with GNK solution. This 0.01% solution was prepared just before use.

11. **Stock 1% Trypsin Solution**

Four gms. of trypsin powder (Difco 1:250) were added to 400 ml. of

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⁵ Note: NaCl and KCl plus phenol red can be combined and autoclaved. Glucose (previously filter sterilized) can be added aseptically e.g. 5 ml. of 20% or 10 ml. of 10%.

⁶ One hundred mgs. of collagenase was received free of charge from Worthington Biochemical Co., Freehold, New Jersey.
1X Phosphate buffered saline (PBS). Since solution was not complete, it was prefilled through ash free filter paper (Schleicher and Schuell #589). The filtrate was then filter sterilized in a Horn filter (D-7 pad) under positive pressure, dispensed in approximately 50 or 100 ml. amounts, sterility tested and stored at -20°C (freezer).

As needed the stock 1% trypsin solution was thawed quickly in a 37°C water bath, diluted 1:4 with Hanks' BSS and the pH adjusted colorimetrically to 7.6-7.8 with sterile sodium bicarbonate solution. The resulting 0.25% trypsin solution was used as the cell dispersing agent in the preparation of kidney cell suspensions and chick embryo cell suspensions.

12. Antibiotic Solutions

a. Penicillin, Streptomycin, Neomycin and Bacitracin Solution.7

- 4 vials of Penicillin G Sodium (1,000,000 units per vial) 4,000,000 units
- 4 vials of Streptomycin Sulfate (1,000,000 ugms per vial) 4,000,000 ugms.
- 4 gms. of Neomycin Sulfate 4,000,000 ugms.
- 0.57 gms. of Bacitracin (40,000 units) 40,000 units

were dissolved in 160 ml. of sterile 1X Hanks' BSS, 40 ml. being used for each antibiotic and the solutions combined. The pH was adjusted after combining the 40 ml. aliquots, the solution was filter sterilized, dispensed in 5-7 ml. amounts (approximately) in screw capped tubes and stored at -20°C.

7Antibiotics were received gratis through the courtesy of Upjohn Company, Kalamazoo, Michigan.
Final concentration is 25,000 units per ml. each of penicillin, streptomycin and neomycin and 250 units per ml. of bacitracin. As needed for tissue culture media and solutions, a tube was removed from the freezer, quickly thawed in a 37°C. water bath and the appropriate amount pipetted aseptically to the solution. The usual amount added to media or solutions was 1 ml. per 100 ml. of solution resulting in a final conc. in the medium of 250 units of penicillin, streptomycin and neomycin and 2.5 units of bacitracin per ml. of solution. In some cases, half this amount was used. e.g. 0.5 ml. stock per 100 ml. solution.

b. Penicillin-Streptomycin Stock Solution

A 1 gram vial of streptomycin sulfate was dissolved by addition of 5 ml. of 2.8% NaHCO₃ solution (sterile), plus addition of 1X Hanks' BSS. The solution was transferred to a sterile prescription bottle and to this was added 1,000,000 units of Penicillin G sodium dissolved in 10 ml. sterile 1X Hanks' BSS and a final volume of 50 ml. achieved. This antibiotic solution was dispensed in 5.5 ml. amounts in white rubber stoppered tubes and the last amount was sterility tested in trypticase soy and MY broth. Tubes were labelled Pen.-Strept. 20,000 U/ml. and stored at -20°C. As needed, a tube was removed from the freezer, thawed quickly in a 37°C. water bath and an appropriate amount pipetted aseptically to the medium or solution. One ml. per 100 ml. of solution was the usual amount used to give a final concentration of 200 units per ml. to the solution.
c. **Mycostatin Suspension**

Mycostatin\(^8\) suspension was prepared by diluting 1 vial of Squibb Nystatin (500,000 units) to 20 ml. with sterile \(\text{ddH}_2\text{O}\). The resulting fine suspension was dispensed aseptically in 2.5 to 3.0 ml. amounts into sterile, white rubber stoppered Wassermann tubes and stored at freezer temperature (-20°C.). It is stable thus for at least two months. Final concentration of the stock solution was 25,000 units per ml.

Mycostatin was not routinely incorporated into tissue culture medium but was available for control of mold growth in tissue culture media and solutions. It was used on several occasions in connection with attempts to produce a cell strain in continuous culture from chick embryo, guinea pig kidney and hamster kidney cells.

13. **Sera**

a. **Calf sera.** Approximately a gallon of young calf's blood\(^9\) (calf not over 6 months old) was collected in a plastic bucket which had been thoroughly rinsed in distilled water and then rinsed with about a liter of sterile PBS in two to three portions, covered with its plastic cover lightly and allowed to air dry. The blood was allowed to clot at room temperature for approximately 2-3 hours, and then was put at refrigerator temperature overnight. (4°C.). The serum was decanted into 250 ml. centrifuge tubes, centrifuged for 20 minutes at approximately 2000 rpm and then decanted to clean 500 ml. érlenmeyers. Approximately 1200 ml.

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\(^8\)Squibb Nystatin received gratis through the courtesy of E. R. Squibb & Sons, 745 Fifth Avenue, New York, N. Y.

\(^9\)Through the courtesy of Mr. Herb Margolis of Arizona Meat Packers, 670 W. 33rd St., Tucson, Arizona.
serum was filter sterilized, sterility tested in MY and trypticase soy broth and stored at -20°C.

b. Rabbit sera. Normal, healthy, large (5 lbs. or larger) prefasted white rabbits were ear bled into open sterile 50 ml. centrifuge tubes. Twenty to 30 ml. of blood per rabbit was collected under clean but not aseptic technique into separate centrifuge tubes. The clots were allowed to retract at room temperature for 2-3 hours and the tubes placed in the refrigerator overnight. The serum was decanted or aspirated, centrifuged at 2000 rpm for 20 minutes to remove erythrocytes, then pooled and filtered through the 100 ml. capacity Horm filter (D-7 pad), dispensed into sterile white rubber lined screw capped 50 ml. centrifuge tubes, sterility tested and stored at 4°C. (refrigerator) or freezer(-20°C.) temperatures.

c. Lamb sera. Sterile lamb serum, for use in tissue culture media was purchased from Difco and stored at refrigerator temperature until used.
B. PREPARATION OF HAMSTER KIDNEY CELL CULTURES.

Hamsters 4-8 weeks of age were ether anaesthetized. The chest and abdominal region were thoroughly swabbed with 70% ethyl alcohol and the animals were bled out by cardiac puncture. Kidneys were removed aseptically and placed in a sterile Petri dish. When the required number of kidneys were thus collected (usually 10) the sterile Petri plate containing the kidneys was taken into the tissue culture room where the tissue was further processed. With sterile instruments, e.g. iris scissors and small forceps, the capsule was peeled off and all other connective tissue and adherant adipose tissue removed. Each kidney was cut into at least 4 pieces and transferred to a beaker containing approximately 50 ml. of Hanks' BSS.

The tissue was rinsed in the Hanks' BSS which was then decanted off. After being rinsed, the tissue was minced into pieces of 2-3 mm. size with scissors and rinsed twice with approximately 25 ml. of Hanks' BSS, each time allowing the tissue pieces to settle to the bottom of the beaker before carefully decanting the supernatant which was discarded. The tissue was then transferred to a 500 ml. trypsinizing flask (Bellco Glass Co.) containing 50 to 75 ml. of 0.25% Trypsin in Hanks' BSS. The pH was adjusted to between 7.4 and 7.6 colorimetrically by addition of sterile NaHCO₃ solution.

A sterile magnetic stirring bar was gently dropped into the flask and the mixture stirred on a magnestir for 20 minutes at room temperature.

Purchased from A. S. Aloe, 140 Beacon St., South San Francisco, California.
temperature. After allowing the tissue pieces to settle for about 5 minutes, the supernatant trypsin solution was carefully decanted and discarded. Fresh trypsin solution was added (100 to 150 ml.) and the mixture stirred overnight in the cold room (4°C.) 16 to 20 hours.

The next morning, the turbid cell suspension was poured through several layers of sterile gauze into a 250 ml. centrifuge tube, centrifuged horizontally in a No. 2 International machine at 300 rpm for 30 minutes.

The supernatant fluid from the packed cells was removed by vacuum aspiration using a sterile 1 ml. pipette. The cells were then resuspended in approximately 100 ml. of Hanks' BSS, recentrifuged at 300 rpm for 30 minutes, the supernatant aspirated off and the process repeated, for a total of two rinses with Hanks' and 3 centrifugings. After removing the fluid from the second washing, the cells were resuspended in approximately 10 ml. of Hanks' during transference to a 15 ml. centrifuge tube.

The 15 ml. centrifuge tube suspension was again centrifuged at 300 rpm for 30 minutes, the volume of packed cells read and the supernatant discarded. An approximate 1:100 dilution of the cells was prepared in outgrowth medium by adding about 10 ml. to the centrifuge tube, pipetting this suspension to about 50 ml. of medium in an erlenmeyer flask and then using the remainder of the 100 e.g. 40 ml. to rinse the remaining cells from the centrifuge tube in several portions. A sterile magnetic stirring bar was gently slid down the side of the erlenmeyer and the cell suspension in outgrowth medium stirred on the magne stir while being dispensed to tubes in 0.8 ml. amounts with a Cornwall auto-
matic pipette. (Becton, Dickinson & Co.) The outgrowth medium had the following composition:

Calf serum 10 ml.
Medium A 88 ml.
Pen. Strep. (20,000U/ml.) 0.5 ml.
NaHCO₃ approx. 1.5 ml. (either 8.8% or 2.8% enough to adjust pH to between 7.2 and 7.4.

The tube cell suspensions were incubated at 36°C. under conditions to be described below for 2 days. An additional 1 ml. of outgrowth medium was then added to each tube and incubation continued for 3 or more days.

Satisfactory growth of the cells could be judged in two ways. The monolayer cell sheet could be observed macroscopically as a thin translucent film adherant to the wall of the tubes, and the pH of the medium was progressively lowered from its initial red toward the yellow due to acid production by the cells in the presence of phenol red.

When cell sheets were judged satisfactory, outgrowth medium was removed completely, the sheets rinsed two times with Hanks' BSS distributed with a Cornwall automatic pipette. One ml. of maintenance medium was then distributed to each tube. After incubation at 36°C. for 24 hours, the cell sheets were checked microscopically under 100X magnification for condition and positioned in racks for viral assay. The maintenance medium had the following composition:
Calf serum 3 ml.
Medium A 95 ml.
Pen. Strep-Neo. 0.5 ml.
Bacit. 9
NaHCO3 to adjust to pH 7.6

Four tubes of cells in good condition, (checked microscopically) were each inoculated with 0.1 ml. viral dilutions. These tubes were incubated at 36°C. unless stated otherwise and examined periodically for CPE microscopically under 100X magnification. An observation track11 placed on the microscope state, permitted manipulation of the round tubes so that the whole cell sheet could be rapidly evaluated under direct observation at 100X magnification.

11Obtainable from Wyble Engineering and Development Company, P.O. Box 223, Silver Spring, Maryland.
C. A METHOD FOR HANDLING TUBE CELL CULTURES.

The method successfully used at the California State Department of Health Viral and Rickettsial Laboratories for monkey kidney, chick embryo and other cell cultures was adopted. This method permits the handling of large numbers of tubes during the processing of cell suspensions from the first dispersal to tubes until after the addition of maintenance medium without the necessity of handling each tube individually.

Stainless steel racks\(^{12}\) designed to each hold 80 test tubes tightly and when placed on its side hold them at approximately a 5° slant were used. Specially washed and rinsed HeLa tubes (ordered with screw caps white rubber lined) were carefully positioned in the rack without caps. The whole rack of tubes was covered with a piece of aluminum foil and then a piece of brown paper cut large enough to cover the rack and come approximately half way down the sides. It was held in place by a string tie. This assembly was autoclaved at 121°C. for 20 minutes. The screw caps or white rubber stoppers for closure of the tubes were sterilized separately, by placing them in Petri dishes, wrapping the Petri dishes in brown paper and sterilizing them in the autoclave.

When the cell suspension in outgrowth medium was ready for dispersal to tubes, the required number of racks so prepared were taken into the tissue culture room and the cell suspension dispersed to the

\(^{12}\)Purchased from Seelye Craftsman Co., 984 Central Avenue, Minneapolis 13, Minnesota.
whole rack of tubes in one operation by means of a Cornwall automatic pipette. This dispersal was accomplished in about 2-4 minutes. The brown string tied paper covering was first removed and discarded leaving the aluminum foil covering over the tops of the tubes. The sterile foil was lifted off gently and placed on a sterile cloth towel during the dispersal process and immediately replaced when it was completed. When the cell suspension was dispersed to as many racks as needed depending on the amount, the racks were put in a simple type of press to be described. This allows incubation of the whole rack of 80 tubes under sterile conditions.

For each press unit two rectangular pieces of \( \frac{1}{2} \) inch plywood were cut (16" x 7\( \frac{1}{2} \)""). At points 3/4 inches from the four corners and 2 center sides, 3/8 inch holes were drilled through both boards. Carriage bolts (6" x 5/16") were counter sunk in one of the boards which served as the bottom of the press. The second board served as the top.

The aluminum foil cover on each rack of tubes was replaced with plastic film which is sterile as it comes off the roll (Saran Wrap). It was carefully unrolled and handled in such a way as not to contaminate the portion that would contact the tops of the tubes.

The plastic film covered rack was placed on the bottom of the press. A rectangular piece of 1 inch foam rubber (not sterile) cut to the size of the Seelye rack was placed on top of the positioned rack and the top board put on and pressed down. By pressing down on the top board with the palm of the hand while screwing wing nuts on until they are tight, the foam rubber was forced down and acted as a satisfactory closure for all eighty tubes at one time. This whole assembly was then
placed in the incubator and required little more space than the rack itself.

After two days incubation, when it was necessary to add 1 ml. of outgrowth medium to each tube, the racks were removed from the presses, taken to the tissue culture room where dispersal of medium was accomplished in the same manner as was the original dispersal of cell suspension. Racks were then reassembled in the press units and reincubated until outgrowth was judged satisfactory (3-4 days).

Outgrowth medium was removed by simply removing the racks from the presses, gently peeling off the plastic film which adheres to the tops of the tubes, and inverting the whole rack over a suitably large discard pan. Since the tubes fit tightly in the racks, they are held from falling out. After about five seconds to allow for drainage, the rack was restored to upright position, and the cell sheets adherant to the walls of the tubes were rinsed by dispersing Hanks' BSS to each tube with a Cornwall automatic pipette, then tilting the whole rack so that the rinse solution gently bathed the cell sheet several times. Rinse solution was removed by inverting the rack as before for removal of outgrowth medium. When the maintenance solution was added after a second rinsing, the white rubber lined screw caps which were sterilized separately were then placed on the tubes which could then be examined individually for condition before addition of the virus inoculum.
D. PREPARATION OF CHICK EMBRYO EXTRACT

Nine to 10 day old embryos were harvested by placing the eggs one at a time in the slight hollow formed from a double thickness paper towel (laboratory paper towel torn in two across so that the two pieces are roughly square). The double thickness paper towel is placed over a short empty can of outside diameter slightly larger than the narrow end of the egg. (An empty frozen juice concentrate can was used).

The egg was placed with the larger diameter end uppermost (air space), painted lightly with a swab of tincture of iodine which was then immediately washed down with a stream of 70% ETOH applied from a squeeze bottle.

A sharp stroke on the shell with a pair of blunt, closed forceps cracked the shell which was removed carefully to nearly the exposed shell membrane. Different sterile forceps and small scissors were then used to tear across the shell membrane to expose the embryo which could be gently lifted up and out with its neck draped over the closed forceps. Adhering membranes were cut away with the sterile scissors and the embryo dropped into a sterile Petri dish.

Ten to 13 embryos were so harvested. Eyes, beaks, legs and wings were removed and the remainder of the embryos were then transferred to a sterile beaker containing about 40 ml. of sterile Hanks' BSS with antibiotics. (Pen. Strept-Neo 250 units/ml. Bacitr. 2.5 units per ml.). The tissue was rinsed and the supernatant fluid decanted off and discarded. The tissue was then minced with sterile scissors, transferred to the barrel of a 50 ml. syringe (without a needle) and forced through into a 15 ml. centrifuge tube. About 10 ml. of tissue pulp resulted which was
transferred to a 50 ml. centrifuge tube. A volume of Hanks' BSS with antibiotics equal to that of the tissue pulp (10 ml.) was added and the tissue suspension centrifuged at 1500 rpm for 20 minutes. The decanted supernatant was the 50% chick embryo extract. Yield was 12-15 ml. This was sterility tested in trypticase soy and MY broths and stored at -20°C.

Immediately before use the 50% extract was thawed quickly by placing the tube in a 37°C. water bath. It was centrifuged at 2000 rpm for 20 minutes and the clear supernatant pipetted to the medium.

Chick embryo extract was used for outgrowth medium for chick embryo cell cultures...4.0 ml. in 100 ml. of medium.
E. PREPARATION OF CHICK EMBRYO CELL CULTURES

The procedure used was the standard procedure used at the California State Department of Health Viral and Rickettsial Laboratories and was as follows:

1. Whole 9 day old embryos were removed to a sterile Petri dish or dishes by the same method as described under preparation of chick embryo extract.

2. Using aseptic technique and sterile instruments, the eyes, beaks, legs and wings were removed from each embryo and the remaining tissue transferred to a beaker containing Hanks' BSS with antibiotics.

3. After all embryos had been so processed the fluid in the beaker was discarded and the embryonic tissue was rinsed two times with fresh Hanks' BSS. The rinse fluid was discarded each time.

4. The tissue was then minced with sterile scissors into pieces of about 3mm. diameter and rinsed three times with fresh Hanks' BSS.

5. The minced tissue was then transferred to the barrel of a 50 ml. syringe (without a needle) and forced through into a trypsinizing flask. More Hanks' BSS was added to the tissue in the flask and the tissue rinsed twice. It was necessary in the last two rinsing procedures to allow more time for the more finely divided tissue to sediment. Approximately 5 minutes with the flask in a slanted position was sufficient. Removal of the supernatant was more efficiently carried out by vacuum aspiration with a sterile pipette.

6. Trypsin solution (0.25%) in Hanks' BSS was then added. The amount depended on the number of embryos processed.
5-10 embryos 200 ml.
11-20 " 300 ml.
21-30 " 400 ml.

7. The tissue suspension was then stirred on the magnet for one hour at room temperature.

8. After stirring, the larger particles were sedimented by placing the flask in a slanting position for about five minutes. The cell suspension was decanted off through several layers of sterile gauze into a 250 ml. centrifuge tube and immediately centrifuged at 600 rpm for 10 minutes.

9. The supernatant was then aspirated off and the sediment was resuspended in Hanks' BSS. The cell suspension was mixed thoroughly and recentrifuged at 600 rpm for 10 minutes.

10. The supernatant was discarded and the sediment was then resuspended in a small amount of Hanks' and transferred to centrifuge tubes, recentrifuged at 600 rpm for 10 minutes and the volume of packed cells recorded.

11. The supernatant was removed by aspiration and a 1:200 cell suspension prepared in outgrowth medium and distributed by Cornwall pipette to tubes or prescription bottles.

12. After 24 to 48 hours incubation (in the press arrangement described for hamster kidney cell culture incubation) the cell monolayers were rinsed twice with Hanks' BSS and maintenance solution was added. Tubes were then checked for condition in preparation for virus inoculation.
F. PREPARATION OF VIRUS INOCULA

Two strains of St. Louis encephalitis virus were received from the California State Department of Health Viral and Rickettsial Laboratories on February 25, 1960. The Winkler strain was received in the form of two sealed tubes of fifth passage mouse brain (M₅) and the Ruis strain as two sealed ampoules of 20% mouse brain in 0.75% BABS. Both were received and kept under dry ice storage conditions during the whole period of laboratory experimentation. The Winkler strain was used exclusively because of available information concerning its performance in mice and hamsters. (Lennette, 1946) One tube containing mouse brain was quickly thawed and opened. A 20% w/v suspension of mouse brain was prepared by hand grinding with alundum in a sterile mortar using sterile 0.75% BABS as diluent. The 20% suspension was distributed in 1 ml. amounts in sterile Wassermann tubes, tightly stoppered with white rubber stoppers and stored at dry ice temperature in small cans covered with plastic film. One ml. of 10% suspension was prepared at this time and stored for 24 hours at freezer temperature. The 10% suspension was used to prepare 1% (10⁻²) and 0.1% (10⁻³) dilutions for intra-cerebral inoculation into mice 4 weeks old. Twelve mice, under light ether anaesthesia, were inoculated intracerebrally (ic), 6 with 10⁻² and 6 with 10⁻³ dilutions. A total of 7 brains were harvested aseptically from mice which developed typical encephalitis symptoms. These mouse brains were stored in white rubber stoppered Wassermann tubes at dry ice temperature.

The 20% suspension prepared and stored as described above and the sixth passage mouse brain material (M₆) were used for tissue culture
inocula, immunization of hamsters and rabbits throughout the entire study. When viral dilutions for tissue culture inocula were prepared, the stored tube was thawed quickly and kept cold (1°-4°C) by placing it in an ice cube tray half filled with melting ice which served as a rack for the dilution tubes. Chilled diluent (BABS) premeasured into the appropriate number of tubes by pipette was used to prepare log dilutions.
SLE mouse brain (M₆) stored at dry ice temperature was used for immunization of hamsters and rabbits as follows: A 20% suspension of SLE infected mouse brain in BABS was prepared from mouse brain stored on 3/7/60. After hand grinding in a sterile mortar with alundum as abrasive and centrifuging at 2000 rpm for 10 minutes to remove the alundum, the supernatant which is the 20% suspension, was distributed into three sterile Wassermann tubes. Two tubes were stored at dry ice temperature. One-half ml. of the 20% suspension was added to 9.5 ml. BABS to yield 10 ml. of 10⁻² dilution. Eight ml. of this preparation was stored at dry ice temperature and 2 ml. added to 8 ml. of BABS to give a 2x10⁻³ dilution. An equal volume (10 ml.) of Bayol F (Penola Oil Company) was added to this in a 50 ml. centrifuge tube and thoroughly shaken to homogenize and provide 20 ml. of a 10⁻³ dilution. Two rabbits were injected subcutaneously (sc) with 2.5 ml. of this emulsion. A total of 10 hamsters, five males and five females, were each injected intraperitoneally (ip) with 0.6-0.8 ml.

One week later the 3 ml. of 10⁻² dilution and one tube of 20% suspension were quickly thawed. Four-tenths ml. of 20% suspension was added to the 8 ml. to provide a 2x10⁻² dilution which was then homogenized with an equal volume of cold Bayol F. Rabbits were injected subcutaneously at the nape of the neck with five ml. each and each hamster received 0.6 ml. intraperitoneally (ip).

Five days after receiving the second ip. injection, one female hamster was moribund with definite symptoms of encephalitis. It was killed by ether and the brain harvested aseptically and stored at dry
ice temperature. The hamsters were not re-injected.

The rabbits were given a third weekly injection of 5 ml. each of 10% suspension of freshly ground mouse brain \( (M_0) \) which had been stored at dry ice temperature. Three weeks later the rabbits were ear bled and serum was obtained by the procedure described for obtaining normal rabbit serum. The serum was stored at refrigerator temperature \( (4^\circ C) \). Both normal and "immune" rabbit sera were inactivated at 56\(^\circ C\) for 30 minutes.
A. STUDIES ON TRYPsinIZATION AND COLLAGENASE TREATMENT OF SPLEEN

For testing of solutions, reagents, media and techniques, guinea pig kidney and spleen cell cultures were prepared by the method described. The spleen was removed aseptically at the time of removal of the kidneys and placed in a separate sterile Petri dish. The spleen processing was run parallel to and was identical to that of the kidney. About 200 ml. of 0.25% trypsin was prepared from the stock 1% trypsin immediately before use. For each run therefore, the same trypsin solution was used, a portion for kidney tissue and another portion for spleen tissue. It was obvious from the first that the spleen tissue did not respond to trypsinization as did the kidney tissue. After comparable overnight trypsinization at 4°C, the kidney tissue suspension was cloudy with dispersed cells while that of the spleen was almost clear. The cell pack yields of spleen and kidney are indicated in Table I. Although the amount of spleen tissue was roughly one third that of kidney, the cell yield was generally about one tenth that of kidney.
TABLE I

Volume of Packed Cells* Obtained from Guinea Pig Kidney and Spleen Tissue

<table>
<thead>
<tr>
<th>Date</th>
<th>Tissue</th>
<th>Cell Yield in ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>11/5/59</td>
<td>Spleen</td>
<td>&lt; 0.2</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>1.1</td>
</tr>
<tr>
<td>11/28/59</td>
<td>Spleen</td>
<td>&lt; 0.13</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>1.0</td>
</tr>
<tr>
<td>13/15/59</td>
<td>Spleen</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>0.2</td>
</tr>
<tr>
<td>2/5/60</td>
<td>Spleen</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*Centrifuged at 300 rpm for 30 minutes

Further attempts to obtain spleen cell suspension by treatment of the spleen tissue with 0.01% collagenase in GNK solution (Hinz and Syverton, 1959) were also unsuccessful. Spleen cell yields obtained by this method are indicated in Table II. Kidney cell yields from the same animal obtained by trypsinization are given for comparison. Various attempts to increase spleen cell yield by pretrypsinization and post treatment of various types did not substantially increase yields and it was finally concluded that spleen cell cultures comparable to kidney cell cultures could not be obtained by the trypsinization or collagenase methods used.
<table>
<thead>
<tr>
<th>Date</th>
<th>Tissue</th>
<th>Pretrypsinization</th>
<th>Collagenase in GNK sol'n.</th>
<th>Cell Yield in ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2/23/60</td>
<td>Guinea Pig spleen none</td>
<td>I* 2 hrs 37C</td>
<td>≤ 0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>kidney†</td>
<td>II overnight 4C</td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td>3/10/60</td>
<td>Guinea Pig spleen 2 hrs 4C</td>
<td>I 2½ hrs 37C</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>kidney</td>
<td>II Hanks' 1 hr RT</td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>3/23/60</td>
<td>Guinea Pig spleen 1 hr 37C</td>
<td>I 1 hr 37C</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>kidney</td>
<td></td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td>3/29/60</td>
<td>Guinea Pig spleen 2 hrs 4C</td>
<td>I 1¼ hrs 37C</td>
<td>≤ 0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>kidney</td>
<td>II Hanks' 16 hrs 4C (some lost)</td>
<td>≤ 0.08</td>
<td>0.3</td>
</tr>
<tr>
<td>4/18/60</td>
<td>3 Hamsters spleen overnight 4C</td>
<td>2+ hrs 37C</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>kidney</td>
<td></td>
<td>&gt;0.12</td>
<td></td>
</tr>
</tbody>
</table>

*I represents crop yield after collagenase treatment

**II represents crop yield after posttreatment of tissue

† trypsinization procedure only was used for kidney tissue
B. CPE OF SLE IN HAMSTER KIDNEY CELL CULTURES

Cytopathogenesis is not directly and immediately apparent in cell cultures by simple observation. Evaluation of CPE is considerably enhanced by experience with the appearance and progressive deterioration of the specific cell system under study and judgement of specific viral degeneration must always be based on comparison with control cultures either uninoculated or preferably inoculated with 0.1 ml. of diluent. The following experiments were designed in order to develop criteria for the evaluation of CPE in the hamster kidney cell monolayer cultures.

**Experiment 1**

Four tubes of hamster kidney cell monolayers were each inoculated with 0.1 ml. of the Winkler strain of SLE in log dilutions from $10^{-1}$ to $10^{-6}$. Four control cultures were inoculated with 0.1 ml. BABS, the diluent, and all were incubated at 36°C. Each tube was examined daily to ascertain the condition of the monolayer which was recorded as - if the condition was good, + if there was some questionable evidence of dead cells and + to +++ indicating progressively greater degeneration. The color of the maintenance solution in each tube was also recorded daily. As acid accumulated, the indicator changed from the original pink through orange and yellow orange to yellow.

After 24 hours incubation, the color of the maintenance medium in most of the tubes was a yellow orange (approximately pH 7.2). The medium from each set of four tubes was pooled in sterile Wassermann tubes, labelled, and stored at dry ice temperature. Fresh maintenance solution was distributed to each culture tube and incubation and readings
continued daily until cytopathogenesis was evident, and the controls deteriorated.

The presence of aggregations of cells which appeared opaque under the conditions of observation was noted and recorded in each tube. Occasionally from these aggregations of cells there appeared outgrowth of fibroblast type cells much as one might expect from an explant. These aggregations were also seen in the control tubes and it was concluded, and subsequently confirmed, that they are not pertinent to specific viral degeneration.

Deterioration of the inoculated cell monolayers beyond that of the controls was first observable at six days after inoculation and was progressively more definite on the seventh and eighth day.

On the eighth day, the maintenance solution from two tubes of each dilution and controls was pooled in Wassermann tubes and stored under dry ice.

Into one set of tubes 0.5 ml. of 1:10,000 neutral red was pipetted and into a second set was added 0.5 ml. of dilute Giemsa. This was done in an attempt to better visualize the degenerate state of the monolayers, the Giemsa staining dead cells while in the second set of tubes the neutral red which is a vital dye, would be taken up by the living cells. All four tubes of each virus dilution tested including the two in each dilution and controls to which dye solution was not added, were read on the ninth and tenth day. Results were discouraging and it was concluded that under the conditions of the test, the addition of neutral red or Giemsa did not facilitate observation of CPE.
Experiment 2

Experiment 2 consisted of three assays (2A-2B-2C) comparable to that described in Experiment 1 on pages 60, 61. Twenty percent mouse brain (M₅) in BAB₃ prepared on 2/29/60 and stored under dry ice was the source of inocula for all three assays. Virus dilutions for assay 2A and 2B were prepared in BAB₃, however, while the virus dilutions for assay 2C were prepared in maintenance solution. Tubes in assay 2A and 2C were incubated in the usual stationary position at a temperature of 31-32°C, while those of assay 2B were incubated in a roller drum (Wyble Engineering Corp.) at a temperature of 37°C. Results of Experiment 2 are indicated in Table III.

**TABLE III**

*CPE of 3LE in Hamster Kidney Cell Cultures
0.1 ml. inoc./tube Winkler M₅ 2/29/60 mouse brain

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Control</th>
<th>10⁻¹</th>
<th>10⁻²</th>
<th>10⁻³</th>
<th>10⁻⁴</th>
<th>10⁻⁵</th>
<th>10⁻⁶</th>
<th>CPD₅₀*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A †</td>
<td>0/4</td>
<td>4/4</td>
<td>4/4</td>
<td>1/4</td>
<td>0/4</td>
<td>0/3</td>
<td>0/4</td>
<td>2.7</td>
</tr>
<tr>
<td>2B †</td>
<td>0/4</td>
<td>ND**</td>
<td>3/3</td>
<td>3/4</td>
<td>0/4</td>
<td>0/4</td>
<td>ND</td>
<td>3.3</td>
</tr>
<tr>
<td>2C ††</td>
<td>0/4</td>
<td>4/4</td>
<td>3/4</td>
<td>1/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Nominator indicates number of tubes showing CPE. Denominator indicates number of tubes inoculated.

*CPD₅₀ calculated by the method of Reed and Muench (1938)
**ND indicates not done
†BAB₃ diluent
††Maintenance solution diluent

Comparison can be made between 2A and 2C where the only variable was the difference in diluent. The hamster kidney cell cultures used for 2B were prepared from the same kidney tissue fragments as those used
for 2A and 2C, but were prepared from the first trypsinization supernatant which is usually discarded presumably because of the low viability and yield of the cells so obtained. The cell cultures used in 2B were all prepared from the same cell suspension and as such could be used for a viral assay. Since the original cell suspension was different from that used to prepare the cell cultures used in both 2A and 2C, they cannot validly be considered replicates of those used in 2A and 2C. Since the 2B assay was incubated at 37°C in a roller drum instead of 31-32°C in a stationary position, it was surprising to find such good agreement in CPD\textsuperscript{50} endpoints.

The CPD\textsubscript{50} was a great deal lower than that reported by Kissling (1957) for the Hubbard and the 904 strain of SL2 which were 7.0 and 8.5 respectively. Although it is not specifically so stated, Kissling's values are probably the CPD\textsubscript{50} per 1 ml and would consequently be lower by one log unit by comparison with those reported here.
C. THE EFFECT OF NORMAL AND "IMMUNE" RABBIT SERUM OUTGROWTH MEDIUM ON THE CPE OF SLE IN NORMAL AND "IMMUNE" HAMSTER KIDNEY CELL CULTURES

Experiment 4

Five normal male hamsters and five "immune" male hamsters of the same age were anaesthetized with ether and bled to death by cardiac puncture. Each group of five was treated separately and the kidneys of the normal group were removed aseptically to a sterile Petri dish before the "immune" animals were sacrificed and so treated. The 10 kidneys from each group were processed separately by the overnight trypsinization method described previously and the cell yield in each case was 1 ml. Two hundred ml. each of outgrowth medium containing normal or "immune" rabbit serum were prepared as follows: 360 ml. of medium A containing 4 ml. of Pen. Strep. Neo. 250,000 U/ml. Bacit. 250 U/ml. was divided into two portions of 180 ml. each in sterile bottles.

Twenty ml. of pooled and filter sterilized normal rabbit serum which had been inactivated at 56°C. for 30 minutes was added to one of the portions. The serum from the rabbits "immunized", according to the schedule previously cited, was also pooled, filter sterilized, inactivated, and added to the second portion. The respective outgrowth media were designated as NR and IR.

The "immune" (kidney cells from immunized hamsters) as well as normal cells (kidney cells from normal hamsters) were suspended in Hanks' BSS to a volume of 10 ml. and thoroughly mixed by pipetting (10 times). Five ml. were then removed and suspended in 50 ml. of NR medium and the remaining 5 ml. suspended in 50 ml. of IR medium. This resulted in four cell suspensions which were distributed to tubes, i.e., normal
hamster kidney cells in normal rabbit serum outgrowth medium (NCNR), normal hamster kidney cells in "immune" rabbit serum outgrowth medium (NCIR) and kidney cells from "immune" hamsters suspended in normal rabbit serum medium (ICNR) and kidney cells from "immune" hamsters suspended in "immune" rabbit serum medium (ICIR). The four racks of tubes were incubated in a press at 36°C. After 48 hours incubation 0.8 ml. of the proper outgrowth medium was added to each tube and incubation was continued for five days at which time the outgrowth medium was removed from all tubes. All cell monolayer cultures were rinsed two times with Hanks' BSS. Maintenance medium containing 97% medium A, 3% calf serum and a final concentration of Pen. Strep. Neo. of 125 U/ml., Bacit. of 1.25U/ml. and Nystatin of 67.5 U/ml. was added to all tubes. After 24 hours incubation at 36°C., all tubes were checked to determine the condition in preparation for virus inoculation. Results of this microscopic evaluation of cultures are in TABLE IV.

TABLE IV

<table>
<thead>
<tr>
<th>Cell Monolayer</th>
<th>Condition</th>
<th>Total No. Examined</th>
<th>NG**</th>
<th>%NG</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC, NR</td>
<td>Fair*</td>
<td>64</td>
<td>26</td>
<td>40.6</td>
</tr>
<tr>
<td>IC, IR</td>
<td>Fair</td>
<td>64</td>
<td>12</td>
<td>18.8</td>
</tr>
<tr>
<td>NC, IR</td>
<td>Good</td>
<td>53</td>
<td>9</td>
<td>17.0</td>
</tr>
<tr>
<td>NC, NR</td>
<td>Good</td>
<td>54</td>
<td>5</td>
<td>9.3</td>
</tr>
</tbody>
</table>

*Cell monolayers "patchy"
**Unacceptable for viral assay (2+or more)

In general, the condition of all of the cell sheets appeared not as good as those of hamster kidney cells grown in outgrowth medium.
containing calf serum. The Winkler strain of SLE (20% mouse brain in BABS 2/29/60) was prepared by dilution in maintenance medium and 0.1 ml. of each dilution, $10^{-1}$ to $10^{-5}$ was inoculated into four tubes in each series. Four tubes in every series were each inoculated with 0.1 ml. maintenance medium and served as controls. All tubes were incubated at 36°C. in a stationary position and read periodically for CPE.

**TABLE V**

<table>
<thead>
<tr>
<th>Dilution</th>
<th>$10^{-1}$</th>
<th>$10^{-2}$</th>
<th>$10^{-3}$</th>
<th>$10^{-4}$</th>
<th>$10^{-5}$</th>
<th>Control</th>
<th>CPD50**</th>
</tr>
</thead>
<tbody>
<tr>
<td>4A (NCNR)</td>
<td>4/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>1.5</td>
</tr>
<tr>
<td>4B (NCIR)</td>
<td>1/4</td>
<td>2/3</td>
<td>2/3</td>
<td>1/3</td>
<td>1/2</td>
<td>0/3</td>
<td>?</td>
</tr>
<tr>
<td>4C (ICNR)</td>
<td>1/3</td>
<td>0/4</td>
<td>1/4</td>
<td>1/3</td>
<td>1/4</td>
<td>0/4</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>4D (ICIR)</td>
<td>3/3</td>
<td>4/4</td>
<td>2/3</td>
<td>2/4</td>
<td>3/4</td>
<td>0/4</td>
<td>&gt; 4.0</td>
</tr>
</tbody>
</table>

*207 mouse brain (M5) 2/29/60 used as virus source.

**Calculated according to the method of Reed and Muench (1938).**

**Experiment 5**

Five normal female hamsters and three "immunized" female hamsters were bled by cardiac puncture and the kidneys removed aseptically. Procedure was identical with that of Experiment 4. Since the cell yield from the normal hamster kidneys was 1.2 ml. and that of the "immunized" animals 0.8 ml. the normal cell suspension was divided into three equal portions instead of two. The third portion of cells was suspended in outgrowth medium containing calf serum (10%). The result of this procedure was five series of tubes, four of which were comparable to those of
Experiment 4. The fifth series (NCNC) was added to test the impression that rabbit serum outgrowth medium produced cell monolayers culture of poorer condition than calf serum outgrowth medium.

Results of evaluation of monolayer culture in preparation for virus inoculation are in TABLE VI.

**TABLE VI**

<table>
<thead>
<tr>
<th>Cell Monolayer</th>
<th>Condition</th>
<th>Total No. Examined</th>
<th>NG**</th>
<th>%NG</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC, NR</td>
<td>Fair</td>
<td>29</td>
<td>3</td>
<td>27.6</td>
</tr>
<tr>
<td>IC, IR</td>
<td>Fair</td>
<td>34</td>
<td>4</td>
<td>11.8</td>
</tr>
<tr>
<td>NC, IR</td>
<td>Good</td>
<td>37</td>
<td>6</td>
<td>16.2</td>
</tr>
<tr>
<td>NC, NR</td>
<td>Good</td>
<td>50</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>NC, NC</td>
<td>Good</td>
<td>56</td>
<td>1</td>
<td>1.8</td>
</tr>
</tbody>
</table>

*Cell monolayers "patchy"

**Unacceptable for viral assay (2+ or more)

The results of Experiment 4 and 5 show good agreement and warrant the conclusion that kidney cell suspensions prepared from hamsters injected as described, differ significantly from kidney cell suspensions prepared from normal hamsters in their ability to produce cell monolayers in normal rabbit serum outgrowth medium. One possible explanation of results might be made by assuming that a virus infected cell population is obtained from the injected hamsters. Under these conditions a substantial proportion of the cells might be unable to proliferate and under conditions of outgrowth, release virus that would infect neighboring proliferating cells. The presence of "immune" rabbit serum in the outgrowth medium would prevent the spread of virus but would not alter the proportion of cells containing intracellular virus. Free
virus, if it were present in the kidney tissue, would be inactivated by the trypsinization process and/or discarded in the several rinsing processes. Free virus in the rabbit serum would obviously be inactivated by the heat inactivation (56°C. for 30 minutes) used for all sera used in outgrowth media.

Because of the rather low \( CPD_{50} \) results in Experiment 4, all five series of tubes in Experiment 5 were inoculated with dilutions of freshly ground mouse brain \( (M_6) \) using maintenance medium as diluent. Mouse brain suspension in BABS \( (M_5) \) was also diluted in maintenance medium and assayed in all but the ICNR series.

Results are given in TABLES VII \( (M_5) \) AND VIII \( (M_6) \).

### TABLE VII

<table>
<thead>
<tr>
<th>Dilution</th>
<th>( 10^{-2} )</th>
<th>( 10^{-3} )</th>
<th>( 10^{-4} )</th>
<th>( 10^{-5} )</th>
<th>Control</th>
<th>( CPD_{50} )**</th>
</tr>
</thead>
<tbody>
<tr>
<td>5D (NCNR)</td>
<td>4/4</td>
<td>1/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>2.7</td>
</tr>
<tr>
<td>5B (NCIR)</td>
<td>4/4</td>
<td>0/3</td>
<td>0/3</td>
<td>ND</td>
<td>0/4</td>
<td>2.5</td>
</tr>
<tr>
<td>5C (ICNR)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0/4</td>
<td>ND</td>
</tr>
<tr>
<td>5E (ICIR)</td>
<td>3/4</td>
<td>0/4</td>
<td>0/2</td>
<td>ND</td>
<td>0/3</td>
<td>2.3</td>
</tr>
<tr>
<td>5A (NGNC)</td>
<td>2/3</td>
<td>1/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>2.4</td>
</tr>
</tbody>
</table>

*20% mouse brain \( (M_4) \) 2/29/60 used as viral source  
**Calculated according to the method of Reed and Muench (1938)

Numerator indicates the number of tubes showing CPE  
Denominator indicates the number of tubes inoculated
TABLE VIII

CPE in Hamster Kidney Cultures Inoc. With 0.1 ml. SLE (M₆)*

<table>
<thead>
<tr>
<th>Dilution</th>
<th>10⁻²</th>
<th>10⁻³</th>
<th>10⁻⁴</th>
<th>10⁻⁵</th>
<th>10⁻⁶</th>
<th>Control</th>
<th>CPD₅₀**</th>
</tr>
</thead>
<tbody>
<tr>
<td>5B (NCIR)</td>
<td>3/3</td>
<td>3/3</td>
<td>2/3</td>
<td>2/3</td>
<td>0/4</td>
<td></td>
<td>&gt; 6.0</td>
</tr>
<tr>
<td>5C (ICNR)</td>
<td>4/4</td>
<td>3/4</td>
<td>3/4</td>
<td>1/2</td>
<td>0/4</td>
<td></td>
<td>6.0</td>
</tr>
<tr>
<td>5C (ICIR)</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
<td>0/3</td>
<td>0/4</td>
<td></td>
<td>&gt; 6.0</td>
</tr>
</tbody>
</table>

*Freshly ground M₆ (3/7/60), in maintenance used as viral source

**Calculated according to the method of Reed and Muench (1938)

As can be seen by a comparison of the calculated CPD₅₀ in Experiment 4 and 5 (Tables V and VII), there is very little agreement. From a consideration of the data in TABLE V from which the CPD₅₀ for Experiment 4 was calculated, it is obvious that the CPE was irregularly obtained and except for the NCNR series, can scarcely be considered as anything but an estimate. The CPE in Experiment 5 indicated in TABLE VII was obtained using the same viral source for inocula (20% M₆) 2/29/60 as was used in Experiment 4. The CPE obtained in the different series in Experiment 5 are in good agreement and the calculated CPD₅₀'s are very much the same as those obtained in the preliminary testing period and reported in TABLE III.

Since no significant differences were noted in CPD₅₀ in the various series in Experiment 5 and this lack of difference was obtained using both a low titer viral source (20% M₆) 2/29/60 and a high titer
virus source (20% M) 3/7/60, it may be concluded that the CPE obtained with graded doses of SLE virus is not altered by immune serum in the outgrowth medium nor by preparing the kidney cell suspensions from hamsters injected as described.

One point worthy of consideration as a possible explanation for the irregular CPE effects obtained in Experiment 4 and indicated in TABLE V is the age of the hamsters and length of elapsed time between the last injection of SLE and sacrifice of the animals for kidney tissue. The last injection of SLE was given when the hamsters were approximately five weeks old. Those used in Experiment 4 were nine weeks old, while those used in Experiment 5 were 15 weeks old.

If one makes the same assumption of a virus infected cell population as was made in possible explanation of the greater number of unacceptable cultures in the ICNR series, a two week period might allow considerable recovery from this state. The kidney cells used in Experiment 5 would thus have a lower proportion of virus infected cells and react more like the kidney cells from the normal hamsters.

The age of the hamsters must also be considered in connection with the production of satisfactory kidney cell monolayers. In general the younger the animal, the better the monolayers cultures produced probably because of the greater growth potential of the cell populations obtained.

The problem in designing and executing Experiments 4 and 5 was to allow sufficient time after injection of SLE virus for reasonable assurance that a state of immunity would be present, yet obtain kidney tissue from as young animals as was possible.
D. STUDIES OF SLE IN CHICK EMBRYO CELL CULTURES

Although Kissling (1957) reported no CPE of SLE virus in chick embryo cell cultures, the work of Buckley (1959) and of Porterfield (1959a, b, c, and 1960), and others made it seem worthwhile to study the effect of SLE virus in this cell system. In addition to providing a fibroblast type monolayer, chick embryo cell cultures possess distinct advantages. The embryonic tissue is more easily and rapidly processed and trypsinized, the cell population has a greater growth potential and the viable cell yield is greater so that dilution of the cell pack yield is approximately 1:200 instead of 1:100 or 1:125 as is the case with hamster kidney cell suspensions. Confluent monolayers are rapidly grown so that the length of time between processing of tissue and viral inoculation of the monolayers is one to three days instead of seven to nine days as is the case with hamster kidney cell culture.

Experiments 3, 6, 7, and 8 were designed to compare the tube method in which the results are read by evaluation of CPE microscopically under 100 X magnification and a modified plaque method (Porterfield 1959a) which depends on altered ability of infected cells to take up the vital stain neutral red and the results of which are read macroscopically.

In order to test solutions, media and techniques and become familiar with the microscopic appearance of chick embryo cell monolayers, the tissue residuum after preparation of chick embryo extract was trypsinized and used. This method is not recommended for several reasons, but the fact that confluent monolayers could be obtained from tissue mince which had been subjected to centrifuging at 1500-2000 rpm for 20
minutes illustrates the high growth potential of such tissue.

Experiment 6

Tissue from 15 12-day old embryos was processed. Trypsinization was carried out for one and one half hours at room temperature. The yield was 6 ml. of packed cells which were suspended in 900 ml. outgrowth medium of the following composition: \( \text{HO}_2 \) 17%, Medium A 30%, chick embryo extract 0.8%, calf serum 2%, antibiotics solution 0.5%.

The cell suspension was kept mixed on a magnetic stirrer and dispersed by Cornwall automatic pipette into tubes which received 2 ml. each, three and one half ounce prescription bottles which received 5 ml. each and flat sided milk dilution bottles which received 10 ml. each. All were incubated at 36°C. approximately 36 hours when inspection macroscopically indicated confluent monolayers had formed.

Outgrowth medium was first removed from the tubes, the tube cell sheets were rinsed with 2 ml. portions of Hanks' each and 1 ml. of maintenance medium was then added. Maintenance medium consisted of Eagle's L medium with glutamine and antibiotics added but buffered with 0.2M Tris (hydroxymethyl) aminomethane instead of \( \text{NaHCO}_3 \).

Outgrowth medium was then removed from the bottles by insertion of a sterile pipette attached to a vacuum trap. The bottle cell sheets were rinsed with Hanks' BSS distributed by means of a Cornwall automatic pipette and removed in the same manner as was the outgrowth medium. Virus dilutions were then prepared and tubes and bottles were inoculated with different amounts of the same dilution.

After the virus inocula were distributed to both tubes and bottles, the tubes were incubated at 36°C. and periodically read microscopically.
for cytopathogenesis. Maintenance medium containing 2% agar was distributed by Cornwall pipette, 8 ml. to big bottles and 4 ml. to small bottles. The agar maintenance medium was prepared by mixing double strength maintenance medium warmed to 42°C, with an equal volume of 4% agar in ddH₂O premelted and cooled to 42°C. After thorough mixing and holding at 42°C, the agar maintenance medium was dispensed by Cornwall pipette. After the agar medium solidified at room temperature, the bottles were incubated at 36°C. After 72 hours incubation, 1:10,000 neutral red solution in Earle's BSS was added by Cornwall pipette, 4 ml. to big bottles, and 2 ml. to small. Inspection of the bottles after four hours contact with the neutral red solution showed nothing and they were left at room temperature for an additional 48 hours before readings were taken. Results of assay of the Winkler strain of SLE 20% mouse brain, M₅ 2/29/60 in tubes are given in TABLE IX.

**TABLE IX**

<table>
<thead>
<tr>
<th>Dilution</th>
<th>10⁻¹</th>
<th>10⁻²</th>
<th>10⁻³</th>
<th>10⁻⁴</th>
<th>10⁻⁵</th>
<th>10⁻⁶</th>
<th>Control</th>
<th>CPD₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>3C</td>
<td>4/4</td>
<td>2/4</td>
<td>2/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*20% Mouse brain M 2/29/60 viral source
†BABS diluent

Definite CPE was obtained in the tubes but the plaque method yielded unsatisfactory results under these conditions. Although there was some evidence of cell sheet destruction, especially in the large bottles, plaques could not be distinguished or counted. It was evident that the cell sheets had dried out probably during the period allowed
for virus adsorption which was 2-4 hours.

Among other inocula used for tube cultures in Experiment 3 were the hamster kidney tissue cultures fluids obtained from Experiment 1 and 2. In Experiment 1, tissue culture fluid 24 hours after virus inoculation had been pooled and stored at dry ice temperature. The fluid had been pooled from 4 tubes inoculated with dilutions of SLE Mouse brain M₅ 2/29/60 10⁻¹ to 10⁻⁶ as well as 4 control tubes. These were labelled for storage as follows....3LE (Winkler) M₅-HKTC₁ 4/28/60, 24 hours 1, which represented the fluid from the four tubes inoculated with 10⁻¹ dilution, SLE (Winkler) Mouse brain M₅-HKTC₁ 4/28/60, 24 hours 2, which represented the fluid from the four tubes inoculated with 10⁻² dilution, etc. These tubes were quickly thawed and 0.1 ml. as well as 0.1 ml. of a 1:10 dilution of each of the 7 tubes, c, 1, 2, 3, 4, 5, 6 was inoculated into 4 tubes of chick embryo cell cultures. Results are indicated in TABLES X and XI.

**TABLE X**

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>1↑</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A*</td>
<td>0/4</td>
<td>0/3</td>
<td>2/4</td>
<td>0/4</td>
<td>0/4</td>
<td>1/4</td>
<td>0/4</td>
</tr>
<tr>
<td>3D**</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>1/3</td>
<td>0/4</td>
<td>ND***</td>
<td>0/4</td>
</tr>
</tbody>
</table>

Total 0/8 0/7 2/8 1/7 0/8 1/4 0/8

*Stationary incubation 95°F. **Roller drum incubation 95°F. ***Not done. 1↑, 2, 3, etc., represent pooled HKTC fluids inoc. with 10⁻¹, 10⁻², 10⁻³, respectively. Denominator indicates number of tubes inoculated. Nominator indicates the number of tubes with CPE.
### TABLE XI

CPE of Diluted (1:10) TC Fluids from Hamster Kidney Cultures on Chick Embryo Cell Cultures

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>1†</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D**</td>
<td>1/1</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
<td>ND***</td>
</tr>
<tr>
<td>Total</td>
<td>5/5</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
<td>7/8</td>
<td>4/4</td>
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*Stationary incubation 95°F. Nominator indicates the number of tubes showing CPE
**Roller drum incubation 95°F. Denominator indicates the number of tubes inoculated
†1, 2, 3, etc., represent pooled HKTC fluids inoc. with $10^{-1}, 10^{-2}, 10^{-3}$ respectively

The undiluted hamster kidney tissue culture fluids produced very little or no CPE, while 1:10 dilutions of these fluids uniformly gave a CPE.

One might suspect contamination of the diluent with virus especially since the controls for 3A and 3D were tissue culture fluids and not diluent. As an argument against this possibility, however, the results of series 3C (Table IX) done at the same time and including a control, indicate that the diluent was not contaminated with virus.

If one were to assume that an interferon-like substance were produced in the original hamster kidney cultures and that it was high in concentration in comparison to virus at 24 hours, (when the fluids were harvested), then the data might be explained. Interferon has been reported to be produced in chick embryo cell cultures, (Isaacs and Burke, 1959), in rabbit kidney cell cultures (Isaacs and Westwood, 1959), and...
in calf kidney cells by Tyrrell. (Isaacs and Burke, 1959)

Although chick embryo cell cultures are rapidly grown, they also rapidly deteriorate. Because of the more rapid accumulation of acid in the maintenance medium and deterioration of the cell monolayers, they are less satisfactory than hamster kidney cell cultures for evaluation of CPE unless one is dealing with a virus such as western equine encephalitis (WEE) virus which produces a CPE usually in about two days.

**Experiment 7**

An attempt was made to assay SLE (20% suspension of mouse brain Mc) which had been stored at -20°C. for two months, in chick embryo cell cultures grown in 10% normal rabbit serum outgrowth medium and "immune" rabbit serum outgrowth medium. Maintenance medium for both series was medium A with 3% calf serum. Both outgrowth and maintenance media were therefore the same as had been used with hamster kidney cell cultures.

In anticipation of rapid accumulation of acid in the cultures, small pieces of sterilized egg shell were put into the bottoms of the tubes when the maintenance solution was dispersed. It was thought that the CaCO₃ in the egg shell would neutralize the acid as it accumulated and thus prevent deterioration of the cell sheets. This was unsuccessful and small additions (0.03-0.06 ml.) of approximately 0.09 N NaOH daily for three days was also unsuccessful. Controls deteriorated to such an extent that evaluation of CPE was impossible.

A factor of possible importance is the stage of development of the embryos from which the chick embryo cell cultures are prepared. In Experiment 6, nine day old embryos were used but these had been
incubated at a temperature of approximately 95°F, instead of 100-103°F, which is standard. Their stage of development was about 30 rather than 36 to 38 which was the developmental stage of the embryos processed for Experiment 3. (Hamilton, 1952)

Such embryonic tissue might reasonably be expected to yield a cell population with a considerably different and probably higher growth potential than that derived from embryos at a later state of development.

Experiments 7 and 8 were designed to test this possibility. Embryos in stages 36 to 38 were used, the outgrowth and maintenance medium was that of Experiment 6.

By using embryos at this later stage of development one obtains more tissue because of the larger size, better response to trypsinization (5.0-6.0 ml. cell pack yield vs. 0.4 ml. cell pack yield) and the cell populations might reasonably be expected to have a lower growth potential and to deteriorate less rapidly.

In addition to these quantitative differences there may be a qualitative difference in the cell populations obtained from embryos of different stages of development which could influence response of the monolayers to a specific virus agent. Sbert (1951) in a study of the antigens of heart, spleen and brain as they appear at different stages of development in the chick, has found complex antigenic changes during differentiation.

Porterfield (1959a) in his work on development of an adequate plaque method for Arbor B viruses presents evidence to indicate that replacement of NaHCO₃ buffer with Tris buffer in maintenance medium
resulted in chick embryo monolayers which remained in a healthy condition for a longer period of time.

Tris buffer was used in Experiment 3 which resulted in a moderately successful tube method assay. Although plaque attempts in Experiment 3 were unsuccessful, evidence suggested procedural techniques as the cause. Both tube monolayer assay of CPE (Experiment 7) and plaque attempts (Experiment 8) were unsuccessful. However, both the stage of development of the embryos and outgrowth and maintenance media as well as the amount and dilution of the cells dispersed to tubes and bottles differed in successful and unsuccessful assays, therefore, one or all of these factors may be important.
CHAPTER V
SUMMARY

A. In preliminary testing of media, reagents and techniques using guinea pigs and hamsters, it was found that spleen cell cultures comparable to kidney cell cultures were unobtainable by either the trypsinization procedure or collagenase treatment.

B. Adequate criteria for evaluation of CPE in hamster kidney cultures infected with SLE virus were developed in over 600 examinations of nearly 100 hamster kidney cell cultures in four separate assays. Hamster kidney cell cultures provide a suitable biological system amenable to controlled variation for study of the CPE of SLE virus. The Winkle strain of SLE produced a definite CPE under the conditions described at about the sixth day after inoculation and no significant differences were noted when maintenance solution was substituted for BABS in the preparation of viral dilutions.

C. Calf serum outgrowth medium produced better hamster kidney monolayer cultures than did rabbit serum outgrowth medium under comparable conditions.

From studies of the CPE produced by graded doses of SLE virus in hamster kidney cell cultures from normal and immunized animals grown in normal and "immune" rabbit serum outgrowth media, the following conclusions have been reached.

1. Kidney cells from hamsters injected intraperitoneally with SLE
virus four and six weeks before being sacrificed, produce significantly fewer cell monolayers when grown in normal rabbit serum outgrowth medium than do kidney cells from normal hamsters.

2. Neither "immune" rabbit serum outgrowth medium nor kidney cells from monolayers of immunized hamsters produced any significant alteration in CPE obtained with the Winkler strain of SLE virus.

D. Studies of the Winkler strain of SLE in primary chick embryo cell cultures have indicated that:

1. State of development of embryos processed for tissue culture, media composition and the number of cells dispersed are all important factors in the production of cell monolayers satisfactory for assay of SLE virus by either the tube CPE or plaque method.

2. Chick embryo cell cultures are less satisfactory for evaluation of the CPE of the Winkler strain of SLE virus than are hamster kidney cell cultures.

3. An interferon-like substance may be produced in hamster kidney cultures inoculated with SLE virus.
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