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THE FATE OF INTRAVENOUSLY INJECTED HERPESVIRUS IN HAMSTERS.

The University of Oklahoma, Ph.D., 1966
Bacteriology

University Microfilms, Inc., Ann Arbor, Michigan

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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

THE FATE OF INTRAVENOUSLY INJECTED HERPESVIRUS IN HAMSTERS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

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Oklahoma City, Oklahoma

1966

THE FATE OF INTRAVENOUSLY INJECTED HERPESVIRUS IN HAMSTERS

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ACKNOWLEDGEMENT

The author wishes to express his indebtedness to L. V. Scott, Sc.D., for his support of this investigation, friendly advice, and assistance in the preparation of this manuscript. Appreciation is also extended to the other faculty members of the Department of Microbiology and to Walter Joel, M.D., Department of Pathology, for encouragement and assistance during these studies.

This investigation was supported in part by U. S. Public Health Service Grant 5 R01 HD 00194-10 from the National Institute of Child Health and Human Development.

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THE FATE OF INTRAVENOUSLY INJECTED HERPESVIRUS IN HAMSTERS

CHAPTER I

INTRODUCTION

The pathogenesis of most viral diseases includes a viremia by which the infectious agent is spread to organs and tissues far removed from the original site of entry. Generalized herpetic infections with involvement of many organ systems can be produced experimentally in animals, and many cases of disseminated visceral disease have been described in humans, particularly in newborn infants. Studies by many investigators have indicated that spread of the herpesvirus to the central nervous system and other organs occurs not only by neural pathways, but also by hematogenous routes.

Few studies have been made on the factors involved in the spread of blood-borne virus. The macrophages of the reticuloendothelial system (RES) have been shown to play a role in the removal of viruses from the blood similar to that known to occur with other particles, both living and inert. Particle size, the presence of opsonins, and size of the inoculum affect uptake of particles. Uptake of viruses is a more complex phenomenon, however, since it involves a dynamic interaction between the host and particles which may have the capability for attaching to, or infecting, host cells. This may result in destruction of the agent, or in

some cases may be a means of spread to other tissues. Thus the ultimate fate of the host, either recovery or disseminated disease and death, may be decided by the response of the fixed macrophages to viral particles present in the bloodstream.

The present research was undertaken to study some of the events which occur when herpesvirus is introduced into the blood of a susceptible nonimmune animal. Experiments were designed to determine (1) the rate of clearance of the virus from the circulating blood, (2) the factors involved in the disappearance of the virus, and (3) the localization and fate of the virus in animal tissues as indicated by titrations of organs and fluorescent antibody staining.

CHAPTER II

REVIEW OF THE LITERATURE

Although the disease, herpes febrilis, was clearly described as a clinical entity by Richard Morton as early as 1694 (Beswick, 1962), the causative agent was not isolated until the early years of the twentieth century. Grüter (1920) inoculated scrapings from a dendritic ulcer of a human cornea onto the corneas of rabbits and demonstrated that a similar lesion occurred in the animals. In some instances encephalitis developed, and the agent could be maintained by rabbit to rabbit passage of infected brain tissue.

Grüter's basic discovery of the transmissibility of herpesvirus stimulated extensive studies of the mechanisms involved in the spread of the agent in animal tissues. Material from lesions of the various clinical forms of human herpetic infections were found to infect experimental animals (Löwenstein, 1919), and the resulting pathologic findings in animals were shown to be like those in the human, including the presence of specific intranuclear inclusions (Lipschütz, 1921). Later, it was observed that inoculation of the rabbit cornea resulted in a drawing of the head toward the affected side and was followed by symptoms of encephalitis. Although the brain contained demonstrable virus, changes in other tissues were not observed, and it was concluded that nerve tissue was particularly susceptible to herpesvirus (Doerr and Vochting, 1920).

Pathogenesis of Herpesvirus

Neural Transmission

Evidence for the neural transmission of virus to the central nervous sytem (CNS) was reported almost simultaneously by two groups of workers. Goodpasture and Teague (1923a) observed that virus inoculated peripherally into rabbits was readily transmitted directly to the CNS along sensory, motor, or sympathetic nerve fibers. They believed that transmission occurred within axons by invasive proliferation of the virus and not by passive transport. Although these workers did not positively rule out spread of the virus by growth in endoneural cells, consideration of this route was discarded since pathologic changes in these cells were not evident.

Further investigations by Goodpasture (1925a, b, c, 1929) of the pathogenesis of herpetic infections in rabbits established the axonal pathway as a route of viral transmission.

Viral transmission through perineural lymphatic spaces as a possible pathway to the CNS was suggested by Marinesco and Draganesco (1923). This concept was later supported by Boyse et al. (1956), who inoculated virus into various peripheral nerves and later injected iodine-labeled, homologous plasma proteins intravenously. The radioactive proteins accumulated preferentially at sites of viral-induced inflammation. Since inflammatory changes were detected outside of axons, viral spread might be explained better on the basis of dispersion through tissue fluids than along axonal pathways.

The spread of herpesvirus along nerve tracts has since been confirmed by other workers, who used various experimental approaches in

different species of animals. Anderson (1940) inoculated virus into embryonated eggs by various routes, and reported that three modes of viral transmission occurred in this system: (1) invasion of the epithelium by direct contact and spread from cell to cell; (2) centripetal and centrifugal transmission along nerves; and (3) hematogenous spread to establish a generalized infection. She found that serial passage increased virulence for the chick embryo; that increased ability to infect mesodermal and entodermal tissues corresponded with a decrease in ectodermal or neuronal susceptibility.

Slavin and Berry (1943a) instilled virus intranasally in suckling mice and reported that transmission to the CNS, as shown by histologic studies, was along trigeminal and olfactory nerves. Beswick (1960) inoculated unadapted virus intraperitoneally into one-day-old mice and produced an ascending myelitis with paralysis of the hind limbs. Passaged strains of virus never produced paralysis and seldom produced histologic changes in the CNS. Conversely, increased viscerotropism was characteristic of the passaged strains. Burnet (1960) described work of Wildy (in press), who presented evidence of centripetal spread of herpesvirus within nerves. He inoculated virus into the pads of the hind feet of mice and followed the upward progression of the virus to the CNS by detailed titrations of nerves serving the injection sites.

Most of these experimental procedures are subject to question since they utilized either gross titrations of tissues, or pathologic and clinical criteria to determine routes of spread within animals. A recent study of the pathogenesis of herpes encephalitis and myelitis has been made with more sophisticated titration techniques coupled with

localization of the viral antigen in tissues by fluorescent antibody staining (Johnson, 1964a). After intracranial injection of virus in mice, the virus dispersed rapidly in the cerebrospinal fluid. This was followed by viral multiplication in the meninges and ependyma with invasion of both neuronal and glial cells in the underlying parenchyma. Inoculation by subcutaneous or intranasal routes resulted in spread of the virus along corresponding peripheral and cranial nerves. Apparently this spread was not within the axon, but resulted from centripetal infection of endoneural cells (Schwann cells and fibroblasts). Antigen was not found in axons even after infection could be demonstrated around the nuclei of corresponding ganglion cells.

Hematogenous Transmission

Although transmission of herpesvirus along neural pathways has been definitely established as a mode of viral dissemination, equally convincing evidence of the role of viremia as a means of spread has been documented. Soon after the original isolation of herpesvirus, Doerr and Schnabel (1921) reported that encephalitis developed in rabbits following intravenous injection of material from herpetic lesions. When blood from these infected animals was inoculated on the scarified corneas of other animals an encephalitis resulted. It was assumed that a viremia was produced after initial corneal infection, and that subsequent infection of the brain resulted from hematogenous transport of the infecting agent. Later, Greenbaum and Harkins (1925) postulated that herpetic disease was a generalized infection in rabbits, and that viral dissemination occurred by way of the bloodstream. This conclusion was reached by demonstrating that blood from infected animals, which had been concentrated

by drying, produced a keratoconjunctivitis in the scarified corneas of rabbits. Remlinger and Bailly (1926) extended these findings by infecting rabbits by various routes of inoculation with serum, defibrinated blood, and whole blood from animals with experimentally produced herpetic infections. Smith (1931) studied the pathogenesis of a strain of herpesvirus which had been adapted to produce specific lesions in the adrenal glands of rabbits. Intravenous inoculation of this viral strain produced characteristic foci of necrosis in both the cortex and medulla of the adrenals of 15 of 17 animals. When virus was inoculated concurrently with herpes antiserum, pathologic lesions could not be detected.

In 1940 the first direct evidence for herpetic infection of the vascular endothelium was described by Anderson in chick embryos. Although dissemination of the virus occurred by other pathways, she demonstrated that hematogenous spread also occurred. Inclusions were found in endothelial cells of large blood vessels and capillaries, which indicated that bloodborne virus had localized and multiplied in these cells. Metastasis of virus to other tissues resulted from these established foci.

Slavin and Berry (1943b) had noted in previous studies that, following intranasal inoculation of virus in suckling mice, specific herpetic lesions were produced in a variety of organs and tissues, in addition to those in the brain. Further studies indicated that organs of the RES were particularly involved. This observation led them to conclude that dissemination of virus had occurred, and that invasion of parenchymal tissue was by growth through the endothelium of the organs.

More recently, Biegeleisen *et al.* (1957; 1958) used two different strains of herpesvirus to produce experimental viremias in rabbits. Virus

inoculated by corneal scarification was recovered regularly from the blood, at varying time intervals, from 24 to 180 hours. Various organs from rabbits dying from herpetic encephalitis were also shown to contain virus. Transplacental passage of the virus to the fetuses of rabbits was reported also: Virus was isolated from fetal tissue homogenates; specific intranuclear inclusions were detected in fetal tissues; localization of the virus within tissues was revealed by fluorescent staining.

Further evidence for transplacental passage of herpesvirus in experimental animals was reported by Rapp and Falk (1964). A variant strain of virus, previously characterized by plaque size and virulence for weanling mice, was inoculated into pregnant mice and hamster. This variant strain (LPV XIII) was recovered from hamster embryos at a time when virus could no longer be detected in maternal blood. Transplacental passage of other variants tested could not be demonstrated in hamsters, nor was such passage of any herpesvirus strain shown to occur in mice.

When the HF strain of herpesvirus was inoculated intravenously into pregnant hamsters early in the gestation period, there was no histological evidence of infection of fetal tissues (Ferm and Low, 1965). The virus produced multiple foci of acute necrosis in the adrenal cortex, smaller areas of necrosis in the liver, and necrotic lesions in the "chorioallantoic placentas" of the mothers. If virus were injected on the thirteenth day of gestation, however, traces of virus could be recovered consistently from fetal tissues.

Other studies implicating blood-borne virus in the pathogenesis

of herpetic infections have been reported recently. Platt (1964) found that virus inoculated subcutaneously into the metatarsal pads of guinea pigs produced local vesicles containing large amounts of virus. Viremia or generalization of infection did not occur in animals inoculated by this route. However, when virus was inoculated intravenously, a generalized infection developed in which eyes, skin, vascular endothelium, adrenals, and brain were affected.

Johnson (1964a) has also reported an effect of route of inoculation on spread of virus in herpetic infections in mice. Following extraneural inoculation, he found that the virus reached the CNS by both hematogenous and neural pathways. After intraperitoneal or intranasal inoculation, virus multiplied in the viscera and produced a viremia. Spread to the brain occurred through invasion of vascular endothelial cells by blood virus with development of infected foci in the tissue surrounding small cerebral vessels.

Although herpesvirus had long been suspected as a cause of human encephalitis, the first case in which the virus was isolated from the brain was reported by Smith et al. in 1941. Viral isolation was made from brain tissue of a four weeks-old child who died with symptoms of acute encephalitis. In addition to demonstrating specific herpetic inclusions in the brain, virus was identified by cross neutralization tests with known herpes strains and antisera. Two years later, a strain of herpesvirus was isolated from the spinal fluid of a patient with meningeal symptoms which suggested a mild case of choriomeningitis (Armstrong, 1943). The clinical histories of these cases led the reporting physicians to conclude that hematogenous dissemination of virus had occurred.

Other proven cases of human herpesvirus encephalitis have been reported in adults (Zarofonetis et al., 1944; Haymaker, 1949). France and Wilmers (1953) described the disease in a newborn infant twin and noted that both twins showed pathologic evidence of herpetic hepatitis.

Generalized skin infections (eczema herpeticum) suggest blood-borne dissemination of herpesvirus by their abrupt onset and distribution of the lesions (Kipping and Downie, 1948; Beale and Hair, 1956).

Hass (1935) described necrotic lesions and intranuclear inclusions which occurred in the liver and adrenals of a premature infant who died two weeks after birth. The observed hepatoadrenal necrosis probably resulted from herpes viremia. Numerous cases of disseminated visceral disease, in which herpesvirus was isolated from various organs, have been reported in support of Hass's findings (Quilligan and Wilson, 1951; Zuelzer and Stulberg, 1952; Pugh et al., 1954; Colebatch, 1955; Brain et al., 1957; Bird and Gardner, 1959; Bird et al., 1963). All of the above cases occurred in premature or newborn infants. Recently, malnutrition has also been incriminated as a factor conducive to disseminated herpetic infections of older children (McKenzie et al., 1959; McKenzie, 1961).

Transplacental infection resulting in disseminated herpetic disease is the explanation offered in several reported human cases in which viral infection was present at the time of birth and no other portal of entry could be determined (Zuelzer and Stulberg, 1952; McDougal et al., 1954; Mitchell and McCall, 1963; White, 1963).

More direct evidence that a viremia occurs in human herpetic infections has been obtained by isolation of herpesvirus from the blood, as reported by Ruchman and Dodd (1950) in the case of a boy with a primary

herpetic lesion in the nose. Specific neutralizing antibody developed in this patient during convalescence. Recently, White (1963) isolated virus from the blood of an infant who died of a fulminating herpetic infection. The paucity of reports of viral isolations from the blood in human infections may have been due to (1) failure to attempt isolations, (2) obtaining blood samples at a time when viremia is no longer present, and (3) the use of virus-inhibiting anticoagulants when collecting blood samples (Nahmias and Kibrick, 1964).

Review of the literature indicates that spread of herpesvirus can occur by a combination of routes depending upon one or more of a variety of factors: differences in viral strains; host factors, such as age, nutritional status, and immunological history; site of entry of the infecting agent. Viremia plays an important role in the pathogenesis of herpetic infections, although few studies have been made on the mechanisms involved in the spread of virus by this route. In an excellent review, Mims (1964) discussed the possible role of the macrophages in the pathogenesis of viral diseases. These cells are widely distributed throughout the tissues and, therefore, come in contact with viral particles early in infection. Macrophages in the sinusoids of the liver and spleen monitor the circulating blood and presumably interact with viral particles as they do with other foreign particles present in the blood. Little is known about clearance of particles from the blood and most of what is known has been obtained from studies on clearance of inert particles. The remainder of this review will be concerned with reported studies on the removal of particles, both living and inert, from the circulating blood of animals.

Studies on Blood Clearance

Non-living Particles

Injection of various dye and carbon particles into animals has long been used to demonstrate phagocytic cells of the RES or to delineate lymphatic or blood vessels. The first attempt to quantitate removal of inert particles from the blood was reported by Drinker and Shaw in 1921. These workers injected colloidal manganese dioxide intravenously into cats, and studied its relative distribution in various organs. Manganese dioxide was used because it was nontoxic, contained no particles greater than one micron in diameter, and could easily be assayed or observed microscopically in the tissues. Manganese could not be detected in the circulating blood after 18 minutes in most of the animals tested. One hour after injection, approximately 90% of the injected material was recovered in the lungs, liver, and spleen (47%, 38%, and 4%, respectively). These studies were later extended to include other animals (dog, rabbit, guinea pig, rat, chicken, and turtle) to determine if species difference occurred (Lund et al., 1921). In all animals except the cat, particles were removed primarily by the liver. In the cat, lungs and liver were equally effective in early removal of particles. Twelve hours after injection, however, particles which were formerly found in the lungs began to appear in the liver.

The clearance and organ distribution of various colloidal particles from the blood have since been studied by a number of workers. Jaffe and Berman (1928) injected a fine fat emulsion (Oleokoniol) intravenously into rabbits and dogs, and noted that the droplets disappeared from the blood within five to six minutes. The fat appeared first in the Kupffer

cells of the liver which rapidly transmitted their fat content to the liver cells. They reported that after the Kupffer cells became saturated, the phagocytes of the spleen took part in fat storage.

The fate of colloidal iron injected into the blood of rabbits was studied by Polson (1928; 1929), who observed that most of the iron appeared in the lungs, liver, and spleen. Iron was present in the lungs as emboli in blood vessels, but it was located within macrophages of the liver and spleen. Later, Penniger and Hutt (1956) also found that iron was taken up in organs of the RES. When large doses were given, increased uptake occurred in the lungs.

Pohle and Ritchie (1934) made histological and roentgenological studies of the liver, spleen, and bone marrow of rabbits injected with colloidal thorium dioxide. Presence of the colloid, as indicated by radio-opacity, appeared within 15 to 45 minutes after injection and lasted in the spleen for as long as 493 days. Histologically, thorium was demonstrable in the macrophages of liver, spleen, and bone marrow; it was scattered in fine granules throughout liver cells.

McClean and Hinrichs (1938) noted that phosphate, added to serum in vitro, combined with calcium to form a colloidal, physiologically inactive compound. The preformed colloidal calcium phosphate suspended in serum was rapidly removed from the blood when injected intravenously. The colloid was also formed in vivo in the rat (Gersh, 1938a) after intravenous injection of calcium chloride. Calcium phosphate formed in the plasma was phagocytized chiefly by macrophages in the liver, spleen, and bone marrow. Further experiments in the dog confirmed that localization of the particles occurred in the liver and spleen, with little uptake by

macrophages of the bone marrow. Considerable uptake of colloidal particles occurred in the bone marrow of the rabbit if large amounts were injected (Gersh, 1938b).

Jones et al. (1944) injected P^{32}-labeled chromic phosphate into the tail veins of mice, and later examined samples of spleen, liver, and lung for radioactivity. Ninety percent of the colloid was found in the liver.

In 1949, Gofman reported that colloids containing radioisotopes of yttrium, zirconium, columbium, and lanthanum should be useful for studying selective localization of the isotopes in organs of animals following intravenous injection. By varying molar ratios of citrate or lactate salts to the oxide or hydroxide of zirconium or yttrium, colloids with different particle sizes could be prepared. Exact size of particles was not determined, but the preparations were roughly separable into relatively large, intermediate, and small particle colloids by centrifugation. Studies on rates of clearance of the preparations showed differences due to the size of the particles (Dobson et al., 1949). Large particle suspensions were rapidly cleared from the blood, and were taken up primarily in the liver and spleen. The rapidly disappearing component represented 90% of the injected colloid, while 10% of the injected particles disappeared more slowly. Reinjection of blood containing this slow component into a second animal was again followed by slow clearance, which indicated that the depressed rate of clearance for this fraction was not due to saturation of the phagocytizing cells. Intermediate sized particles disappeared more slowly from the blood, and were taken up in large amounts by the bone marrow, as well as by the liver and spleen.

Similar results were reported when radioactive gold colloid was injected into the dog (Sheppard et al., 1951). The half-time for rate of disappearance of this colloid was one and one-half minutes. A very low residual level of radioactivity was reached in about 10 to 20 minutes and persisted for some time. Separation of the various blood cell components showed that radioactivity was associated with the buffy coat fraction of the blood. The persisting or uncleared fraction of colloid was believed to result from adsorption of the particles to blood cells preventing their uptake by reticuloendothelial cells.

Zilversmit et al. (1952) studied the effect of particle size on clearance of radioactive gold colloids in dogs. Particles 40 mμ in diameter were removed more rapidly than smaller ones (10 to 27 mμ). The liver and spleen took up 80 to 90% of the injected particles regardless of their size. When logarithms of the concentration of particles were plotted against time, a biphasic curve was obtained. If the rate of removal of the gold particles had been proportional to the concentration in the blood, a straight line curve should have been obtained with a semilog plot. The data suggested that saturation of the phagocytic cells had occurred. Repeated injections of colloid into the same animal were all cleared, however, and this would indicate that there was no blockade of the RES. The authors postulated that persistence of the slower disappearing colloidal fraction was due possibly to the presence of small gold particles in the inoculum which were removed more slowly than the large particles; or alternatively, to adsorption of the particles to blood cells.

Schoenberg et al. (1961) reported that injection of polystyrene latex particles into rabbits also resulted in a small uncleared fraction

in the blood. They pointed out that nonhomogeneity of particle size probably was not a factor; their particles were known to be uniform in size. Histologic study of organs showed that only a small percentage of the reticuloendothelial cells contained particles regardless of the number injected. Incomplete clearance was thought to be due to constant renewal of the phagocytic cells, with alternate release and uptake of particles from the circulating blood.

A series of quantitative studies on factors influencing clearance of carbon particles were reported in 1951 (Halpern et al.; Biozzi et al.). In initial studies these workers used a carbon suspension which was stabilized in shellac. Results in these experiments were influenced by the toxicity of the stabilizing agent, which caused coagulation of fibrin in vivo when high doses were administered. When, in further studies, a stable and nontoxic carbon suspension of known particle size was injected intravenously into rats, 90 percent of the injected material was removed by phagocytes of the liver and spleen (Halpern et al., 1953; Biozzi et al., 1953). If logarithms of the concentration of carbon were plotted against time, the clearance curve formed a straight line and could be expressed by the equation:

$$\frac{\log C_1 - \log C_2}{T_2 - T_1} = K \qquad (1)$$

in which C_1 and C_2 were carbon concentrations at T_1 and T_2, respectively, and K (the phagocytic index) was a measure of the rate of clearance of carbon particles by the RES for a given dose of carbon. The phagocytic index was shown to be inversely proportional to the dose according to the

formula:

$$K \times D = \text{constant} \qquad (2)$$

when K is the constant from equation (1) and D is the dose of carbon injected. Using this quantitative method of evaluation, the workers showed that the factors which influenced the clearance of carbon from the blood were the quantity injected (size of the dose), and the degree of saturation of cells by the phagocytized particles. It was further demonstrated that previous injections of carbon slowed the clearance of subsequent doses, as indicated by decreasing values for the phagocytic index. Differences in phagocytic activity among animals species, injected with identical doses of carbon, were shown to be due to differences in the relative sizes of the livers and spleens. A correction factor to compensate for different rates due to organ size was derived, and this corrected phagocytic index (α) could be expressed by the equation:

$$\alpha = \frac{W}{Wls} \sqrt[3]{K} \qquad (3)$$

where W is the total body weight, Wls is the combined weight of the liver and spleen, and K is the phagocytic index obtained in equation (1). Experimentally, the phagocytic index (K) was varied with the cube of the ratio $\frac{W}{Wls}$ although the reason for this relationship is not known. The clearance of carbon in rats was later confirmed for other colloids (saccharated iron oxide, chromium phosphate, pigeon erythrocytes, albumin-globulin complexes) in different species of animals (Benacerraf et al., 1957).

Other factors have been shown to influence the rate at which colloids are removed from the blood. Brauer et al. (1956), working with

perfused rat livers, noted an inverse correlation between the velocity of blood flow past the phagocytes and the efficiency of removal of colloids from the blood by the phagocytic cells. These findings were in agreement with results obtained in living animals by Dobson and Jones (1956). They found the blood perfusion velocity in man to be 1.0 to 1.2 ml/gm/min., with an average efficiency for chromic phosphate uptake of 85%. In the mouse the perfusion velocity was higher (1.4 to 1.7 ml/gm/min.) and the efficiency of uptake was lower (70 to 75%).

An effect on clearance of agents used to stabilize colloidal suspensions has been noted by some workers. Murray and Katz (1955) reported that increasing concentrations of gelatin used as a stabilizing agent resulted in decreasing rates of clearance of a constant number of gold particles. They speculated that the decreased rate might be due to the formation of a gelatin-gold-plasma complex which interfered with particle uptake. Further investigation revealed that gelatin alone was phagocytized by Kupffer cells in locations in the liver lobules identical to those taking up gelatin-stabilized colloid (Seaman and Murray, 1956).

Serum factors as <u>in vitro</u> requirements for optimal phagocytosis by vertebrate cells had been pointed out by other investigators (Fenn, 1921; Nelson and Lebrun, 1956; Hirsch and Strauss, 1964). The nature of these factors and their function in phagocytosis <u>in vivo</u> have not been studied until recently. Jenkin and Rowley (1961) investigated the clearance of both bacteria and other colloids in mice and reported that both kinds of particles required serum factors (opsonins) for phagocytosis. A depletion of serum opsonins was demonstrated in mice following injection of a large dose of colloid. They suggested that the reduced rate

of clearance of a second dose of colloid or bacteria was due to lack of serum factors, since addition of serum or plasma produced normal clearance rates. Biozzi et al. (1963) repeated these experiments and reported that they could not find evidence that serum factors either altered the rate of carbon clearance or removed a carbon-induced "blockade" of the RES. Results of their experiments indicated that saturation of the phagocytic cells of the RES was the principal mechanism involved in reduced carbon clearance.

Recently, Normann and Benditt (1965a; 1965b) reinvestigated the clearance mechanism by measuring, in rats, the induced inhibition of clearance caused by the competitive action of two different colloids. They found that a reduction in the rate of carbon disappearance occurred when heat-aggregated albumin was administered during the course of carbon clearance. This inhibition was eliminated when the carbon particles were pretreated with homologous serum. Serum factor(s) which prevented inhibition of clearance was shown to be limited in quantity and could be selectively adsorbed on carbon. It was also discovered that the ratio of carbon to serum was critical, and this fact might explain the different results obtained by other workers. They concluded that any concept of RES blockade must take into account that diminished capacity of the system for phagocytosis may be due either to cell saturation, or to a limitation in available serum components, or both.

Bacteria

The rapid disappearance of living bacteria from the bloodstream has been observed since the early days of bacteriology. Wyssokowitsch (1886) demonstrated that when species of pathogenic and non-pathogenic

bacteria were injected intravenously into rabbits, they were quickly stored in the liver, spleen, and bone marrow. Histologic examinations of organs following injection of Micrococcus tetragenus and the typhoid bacillus indicated that they were taken up in the endothelial cells of these organs. Later, Kyes (1916) injected suspensions of the pneumococcus into pigeons, a species insusceptible to infection with this organism. He noted that the bacteria were rapidly cleared from the blood and localized in the liver and spleen within fixed phagocytes, where they were rapidly destroyed. Bull (1915) reported that the typhoid bacillus was rapidly agglutinated and removed when injected into the bloodstream of rabbits. The clumped bacteria accumulated in the liver and spleen where they were digested and destroyed by phagocytes. These findings were confirmed by Bartlett and Ozaki (1916) who injected Micrococcus aureus intravenously into dogs. The same year, Manwaring and Coe (1916) perfused pneumococci, suspended in immune and nonimmune sera, and Ringer's solution, through isolated rabbit organs. They reported the presence of an "opsonin or bacteriotropin in anti-pneumococcus serum which caused the bacteria to adhere to endothelial cells lining hepatic capillaries." This phenomenon was not observed in the perfused lungs, kidneys, or intestines of the animals. The fate of hemolytic streptococci following intravenous injection in susceptible (rabbit) and nonsusceptible (cat) animals was studied by Hopkins and Parker (1918). The bacteria quickly disappeared from the circulating blood of the cat and were found predominantly in lungs and to a lesser extent in the liver and spleen. Streptococci injected into the rabbit were also promptly removed from the circulation, but were distributed in the organs in different proportions. The liver and spleen took up as many bacteria

as the lungs and many were localized in skeletal muscle.

Factors which influence the disappearance and effective removal of bacteria have been studied by numerous investigators. The virulence of the injected bacteria has been shown to be important. Wright (1927) showed that virulent pneumococci were removed rapidly from the blood of rabbits, but within a short time there was an increase in numbers recovered, presumably due to the ability of the strain to multiply in the tissues and to reinvade the blood. These observations have been confirmed by various other investigators for different bacteria and were reviewed by Wilson and Miles (1964).

Disappearance of injected bacteria is accelerated by the presence of specific antibody. Wright (1927) noted that immunization enhanced removal of the pneumococcus from the circulating blood of rabbits and prevented the appearance of a secondary bacteremia. He also observed that prior contact with the organism stimulated clearance rates in the animals before specific antibody could be detected. Cannon et al. (1932) confirmed this observation by using living staphylococci and paratyphoid bacteria. These workers reported that phagocytosis and intracellular digestion of the organisms were enhanced by immune serum. Later studies by Sullivan et al. (1934), with the same organisms, showed that the degree of localization of the injected bacteria in different organs was not significantly altered by immunization, but that more rapid removal did occur. Teale (1935) reported that virulent bacteria to which the normal animal had no detectable antibody and was highly susceptible were rapidly cleared from the circulating blood. On the other hand, Klebsiella pneumoniae was reported to be cleared more rapidly in dogs immunized both

actively and passively, than in normal animals (Kerby et al., 1950). Later, it was shown that a nonmucoid variant of Friedlander's bacillus was removed more rapidly than encapsulated strains of the same species (Kerby, 1950).

Normal human, rat, and mouse sera were found to exert an opsonic effect upon phagocytosis of Escherichia coli in the perfused rat liver (Howard and Wardlaw, 1958). Studies using heated serum, serum absorbed with homologous organisms, and zymosan absorbed serum indicated that the opsonic serum factors were specific antibody, complement, and another heat-labile factor which probably was properdin. A comparative study of various species of bacteria was reported by the same workers a year later (Wardlaw and Howard, 1959). Seven Gram-negative and seven Gram-positive bacterial strains were studied by perfusion through isolated rat livers. Three flagellated, Gram-negative strains suspended in Ringer-Locke solution were taken up by the liver, whereas the three nonflagellated, Gram-negative strains were not. All of the Gram-negative strains were phagocytized if they were suspended in normal serum. Heating at 56 C. reduced the opsonic activity of the serum for all of the Gram-negative strains. Four Gram-positive strains were readily phagocytized from Ringer-Locke solution, but both heated and unheated serum reduced phagocytosis. Two clostridial strains behaved as Gram-negative strains, and an encapsulated pneumococcus was not removed, into the tissues, from either Ringer-Locke solution or serum suspensions.

In 1959, Benacerraf et al. made a study of the clearance of Staphylococcus aureus and Escherichia coli in mice using the quantitative methods which they had developed for the study of phagocytosis of

inert colloidal particles. Clearance of P^{32}-labeled heat-killed E. coli and staphylococci was found to be exponential in respect to time, and the bacteria were taken up primarily by the liver and spleen. Staphylococci were removed at a maximum rate in mice which had average serum agglutinin titers of 1:32, and the efficiency could not be improved by increasing specific antibody. The rate of clearance of E. coli, however, was directly related to the concentration of specific agglutinins in the blood. Normal mice with low E. coli agglutinating titers cleared the organism slowly, and the rate of clearance could be increased to a maximum by the addition of either specific antiserum or pretreatment of the mice with heterologous endotoxin. Enhanced clearance of E. coli could also be transferred to normal mice by the serum of endotoxin-treated mice.

Jenkin and Rowley (1961) injected both virulent and avirulent unopsonized strains of Salmonella and Klebsiella, intravenously, into mice and followed their clearance from the blood. All avirulent strains were removed at a faster rate than were the virulent strains. Treatment of the virulent strains with pig serum which had a high titer of opsonins, even though unrelated to specific O antibody, greatly increased the clearance rates. Virulent strains treated with mouse anti-lipopolysaccharide serum also enhanced clearance rates, but to a lesser degree.

Recent studies indicate that the RES is functional at an early age; rates of clearance remaining stable from time of birth to weaning, when increased phagocytic function occurs, (Reade and Jenkin, 1965). After carbon injection, histologic studies showed that distribution and uptake of particles in macrophages of fetal rat organs was similar to that found in adults (Reade and Casley-Smith, 1965).

Plant and Bacterial Viruses

Investigations of the clearance of viruses from animal blood are fewer than those which have been reported for inert particles or bacteria. Many of these studies have been made with plant viruses or bacteriophages which obviously lack the ability to multiply in animal cells and, therefore, provide little information concerning viral pathogenesis. Since plant and bacterial viruses are comparable to many animal viruses in size and, in most cases, are relatively simple to assay, they have been useful in studying physical interactions between viruses and animal cells. Smirnow and Goldin (1931) injected Shigella bacteriophage intravenously into guinea pigs and 24 hours later recovered the phage, in low concentrations, in the blood, liver, spleen, and kidney. Tissue examination for phage was not attempted prior to 24 hours, which possibly accounted for the reported low recovery rate. Nungester and Watrous (1934) demonstrated that staphylococcal phage injected intravenously into rats accumulated in the spleen and liver to the same general degree as bacteria. Two hours after inoculation, the organs contained greater amounts of viable phage particles than did the blood.

The fate of Bacillus megatherium bacteriophage inoculated intraperitoneally into mice was investigated by Keller and Engley (1958). Three hours after inoculation there was a decrease of 90% in the number of phage particles present in the blood. Twelve hours after inoculation when phage particles were no longer recoverable from the blood, lungs, kidneys, and brain, particles could be found in the liver and spleen. Phages introduced orally were also consistently recovered from the circulating blood. Results suggested that particles of this size could enter

directly into the blood through the capillary endothelium and be concentrated in the organs of the RES. In a later study, using the same phage injected intravenously into dogs, Keller and Zatzman (1959) reported that the rate of disappearance was rapid for the first 15 minutes, and at 20 minutes declined to a more gradual rate of disappearance which persisted until termination of the experiments at 90 minutes. Factors affecting disappearance of the bacteriophage from the blood were postulated to be (1) plasma inhibitors, (2) nonspecific adsorption to cell surfaces, (3) excretion of particles into extracellular fluids. That uptake by organs of the RES was also a factor in the disappearance of the particles was indicated by a concentration of phage particles in the liver and spleen 196 to 873 times greater than that found in the blood plasma at the same time.

Sulkin et al. (1957) reported that normal rabbit serum inhibited staphylococcal phage in vitro; inhibition could be reversed by heating serum or adsorbing it with zymosan. Clearance rates of rabbits which were pretreated with zymosan four hours prior to intravenous inoculation of phage were three- to tenfold higher than those of untreated animals. In this work, it was not determined whether the effect of zymosan on clearance was due to blockade of the phagocytic cells or to direct neutralization of the inhibitory plasma fraction.

Localization of tobacco mosaic virus in the organs of the RES after intravenous inoculation into animals has also been shown to occur (Gavosto and Ficq, 1953; 1954). Virus labeled with C^{14} was found primarily in the liver and spleen, with lesser amounts present in lymph nodes and kidneys. Virus was taken up by macrophages in the red pulp

of the spleen, and was found in both Kupffer cells and parenchymal cells in the liver.

Animal Viruses

In early studies on the pathogenesis of poliomyelitis and smallpox, experimentally produced viremias were studied as possible routes of transmission. Flexner and Amoss (1912) injected poliovirus into the blood of monkeys and noted that virus appeared rapidly in the spleen and bone marrow, but was not detectable in kidneys, spinal fluid, or brain. Clark et al. (1914) reported that when large and overwhelming quantities of poliovirus were injected into monkeys, the virus persisted in the blood for at least 72 hours and could lead to disease. After 10 days, when paralytic symptoms developed in the animals, virus could no longer be detected in the blood. After intravenous injection of vaccinia virus into rabbits, lungs, liver, spleen, and adrenals were the organs most commonly infected; virus was irregularly recovered from the blood up to six days after injection (Douglas et al., 1929). Later, Smith (1929) reported that vaccinia virus inoculated intravenously into rabbits was rapidly fixed by the formed elements of the blood. Fractionation of the blood after inoculation showed that the virus was attached to either white cells or platelets and could not be detected in whole blood. Masking of viral presence was said to be due to antibody formation as early as the second day following inoculation. Plasma antibody apparently did not affect cell-fixed virus since washed cells could initiate infection in other animals.

During studies on Rift Valley fever virus in mice, Mims (1956a, b) found that virus inoculated into the bloodstream was quickly adsorbed by susceptible cells; 75% of the inoculum disappeared within 30 minutes.

When the virus disappeared from the blood it was no longer detectable in other tissues, even when the entire mouse carcass was examined. Within 5 to 7 hours one growth cycle of virus had occurred in cells, and the newly released virus was taken up by uninfected cells. Virus did not reappear in the circulating blood until further growth cycles infected all cells. If sufficient doses of virus were given, so that all susceptible cells were infected at once, virus appeared in the blood in high titer after one growth cycle.

Later, in studies on ectromelia virus in mice, Mims (1959a) found that 90% of the intravenously injected virus was removed from the blood within 2 to 3 minutes. Titration of organs five minutes after viral inoculation showed that 95% of the virus was present in the liver, whereas less than 4% could be found in the spleen, and only 0.3% of the injected virus remained in the circulating blood. Virus was shown to be present in the macrophages lining the liver sinusoids by fluorescent antibody staining, but none of the hepatic cells appeared to contain virus. A small proportion of virus, which persisted in the blood after most had been taken up by phagocytic cells, was associated with circulating platelets or leucocytes and therefore could not be removed from the blood by macrophages. Further studies on the growth of the virus in the liver (Mims, 1959b) indicated that hepatic cells were not infected until virus was released following a cycle of growth in the macrophages. New virus from these cells later infected neighboring liver cells. After three or more stepwise increases in the size of the infected foci, when almost all of the liver cells were infected, the mice died. Several viruses of smaller size than ectromelia were also tested to determine if

particle size affected uptake of the virus. All of the viruses tested were taken up by the littoral cells of the liver and none of the particles reached hepatic cells during the time of observation. Clearance and uptake of poliovirus was slower than other viruses tested, possibly because of its smaller particle size. A subsequent study of influenza virus (Mims, 1960) showed that, although this virus was taken up by macrophages in the liver, it did not grow in these cells or infect hepatic cells unless massive doses of virus were injected. Hepatic cells, however, were readily infected when the macrophages were by-passed by injecting virus directly into the liver via the biliary tract.

The kinetics of blood clearance of two P^{32}-labeled viruses, vesicular stomatitis and Newcastle disease, were studied by Brunner et al. (1960). Both viruses were cleared rapidly when injected into mice. In the first stage of clearance most of the radioactive particles disappeared with a first order rate. This was followed by a second stage of residual radioactivity which circulated for a longer period of time. This persisting activity was thought to be due to smaller or less phagocytizable particles. Most of the labeled viruses were recovered from the liver, with smaller amounts being recovered from lungs, spleen, and kidneys. Blockade of the RES with thorotrast slowed the rate of clearance, with over 60% of viral activity still present in the blood at 20 minutes and an associated, great decrease in liver uptake. Incubation of Newcastle disease virus with antiserum before injection accelerated clearance significantly, and viral uptake by the liver was increased. In mice injected with antiserum prior to viral inoculation, the initial rate of clearance was the same as that observed in control mice. Blood concentrations measured 15 to 20

minutes after injection of virus, however, were significantly lower in the antiserum treated mice.

An inhibitory effect of a corticosteroid upon the clearance of poliovirus from the circulatory system of the mouse has been described (Byatt and Rasmussen, 1964). Decreased rates of viral clearance were reported to occur in prednisolone-treated mice. This was associated with a marked increase in viral uptake in the spleen despite the fact that spleens in treated mice were significantly smaller than those of untreated animals.

Studies of herpesvirus clearance have not been reported other than one by Geller et al. (1953) who worked with eleven human volunteers. These were patients who all had various neoplastic diseases, as well as measurable levels of anti-herpetic antibodies in their serum. No significant amount of virus was recovered from their blood following intravenous injections of large amounts of herpesvirus. Failure to recover virus from these individuals was possibly due to one or more of the following factors: (1) presence of specific antibodies in the blood; (2) a nonspecific, heat-labile antiviral factor demonstrated in the plasma of one individual; (3) removal of virus by organs of the RES. Indirect evidence for RES uptake was indicated by a noticeable rapid rise in neutralizing antibody levels in some of the subjects following intravenous injection of virus. This response suggested that antigen must have remained available to the body as an antigenic stimulus for a period of time.

CHAPTER III

MATERIALS AND METHODS

Virus Strain

The HF strain of herpes simplex virus (HSV) (Flexner and Amoss, 1925) used in these studies was received from the Communicable Disease Center, Atlanta, Georgia, and has since been maintained in this laboratory through numerous passages in HeLa cells. This cell-adapted strain was used in the production of specific hyperimmune serum in rabbits. Virus was passed three times in the yolk sac of eight to ten day-old embryonated eggs for growth adaption prior to preparation of the viral pools used in hamster clearance studies.

Animals

Adult male, random bred hamsters (Cricetus cricetus), obtained from the Lakeview Hamster Colony,[1] were used in all clearance studies. Animals varied in size from 110 to 150 grams with an average weight of 130 grams, and were fed a diet of mouse breeder pellets and water in this laboratory. Newly obtained hamsters were allowed to adjust to their new environment for one to two weeks before they were used experimentally.

Young albino rabbits which weighed approximately 2 kg and were

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[1]Newfield, N. J.

obtained locally, and were used for the production of antiserum.

Tissue sections for titration of fluorescein labeled antiserum were prepared from three to five day-old suckling mice of the Webster stain from an inbred colony maintained at this institution.

Embryonated Honegger white Leghorn eggs which had been incubated for nine to eleven days were used for preparing chick embryo cell cultures and embryo extract. Viral pools employed in clearance studies were prepared from extraembryonic fluids of seven to nine day-old embryonated eggs.

Reagents

Deionized galss-distilled water was used for the preparation of all solutions and media and served as the final rinse for all glassware. Reagent grade chemicals were employed in the preparation of all solutions unless otherwise designated.

Balanced salt solutions for the preparation of cell culture media, viral diluents, and tissue rinses were prepared as formulated by Hanks and Wallace (1949), or by Earle et al. (1943). Hanks' (HBSS) or Earle's (EBSS) balanced salt solutions were made by diluting stock concentrated solutions in distilled water before they were sterilized by positive pressure filtration through Seitz filters[2].

Phosphate-buffered gelatin saline (BGS) for the preparation of virus dilutions, in some instances, was prepared as described by Scott, (1956).

Since heparin has been reported to combine with HSV and thus

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[2]Seitz EK sterilizing filter sheets. Republic Seitz Filter Corp., Milldale, Conn.

prevent attachment to cells (Nahmias and Kibrick, 1964), the possible inhibitory effects of sodium citrate and ethylene diamine tetra-acetic acid, disodium salt (EDTA), anticoagulant compounds were tested. Solutions containing sodium citrate proved to be the most suitable for experimental use. Growth medium (HLY-10, to be described later) or 0.85% saline containing 2% sodium citrate as an anticoagulant were used for collecting blood samples.

A 2% stock solution of trypsin[3] was prepared according to the method of Wallis et al. (1961) filter sterilized, and stored in convenient amounts at -25 C. Working solutions (0.1 or 0.2%) were prepared by diluting stock solutions in sterile calcium and magnesium free buffer for use in the preparation of primary cell cultures and for dispersing cell monolayers.

Chick embryo extract (EE50) was either obtained commercially[4], or prepared according to the method of Schmidt (1964). Extracts were stored at -25 C until needed. After thawing, the extract was clarified by centrifuging at 1000 x g for 10 minutes.

Penicillin[5] and streptomycin[6] stock solutions were prepared in sterile distilled water and stored at -25 C in one ml amounts. They were added as needed to media and reagents to provide a final concentration of 200 units of penicillin and 200 mcg of streptomycin per ml.

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[3]Trypsin 1:250, Difco Laboratories, Detroit, Mich.

[4]Microbiological Associates, Bethesda, Md.

[5]Potassium penicillin G, Eli Lily and Co., Indianapolis, Ind.

[6]Streptomycin sulfate, E. R. Squibb and Co., New York, N. Y.

Newborn calf serum[7] was inactivated by heating at 56 C for 30 minutes and stored at -25 C. The serum was thawed, sterilized by Seitz filtration, and added in appropriate concentration to tissue culture media or viral diluents. Hamster serum was prepared from blood obtained by cardiac puncture. The blood was allowed to clot at room temperature and was placed at 4 C for clot retraction. The serum was collected and centrifuged at 1000 x g for 15 minutes to remove the cells. The clarified serum was inactivated at 56 C for 30 minutes, filter sterilized, and stored at -25 C.

Tissue Culture Media

HeLa cells were grown in a medium (HLY-10) containing 0.5% lactalbumin hydrolysate[8], 0.1% yeast extract[9], and 10% inactivated calf serum suspended in HBSS. When monolayers were complete, cells were maintained in a lactalbumin hydrolysate-yeast extract medium in EBSS containing 5% inactivated calf serum (ELY-5).

Pig kidney cells (PK) were grown in the chemically defined minimum essential medium of Eagle (1959), supplemented with added nonessential amino acids[4] and 10% inactivated calf serum in Hanks' solution (HMEM-10). Cells were maintained in a similar medium which contained only 5% serum and EBSS as a base (EMEM-5).

Tube cultures of primary chick embryo cells were grown in a

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[7]Lot #27463B, Grand Island Biological Company, Grand Island, N.Y.

[8]Nutritional Biochemical Corporation, Cleveland, Ohio.

[9]Difco Company, Inc., Detroit, Mich.

lactalbumin-yeast extract outgrowth medium in HBSS containing 2% calf serum and 4% EE50. The maintenance medium used for chick embryo tube cultures was HMEM without serum.

Cell Lines and Propagation Procedures

Continuous Cell Cultures

Continuous-passage cell cultures were used in certain phases of these studies. A strain of cells derived from an epitheloid carcinoma of the human cervix (HeLa) originally isolated by Gey et al. (1952), was obtained from the Carver Foundation, Tuskegee, Alabama, in 1962. It has been maintained by weekly passage in this laboratory since that time.

A continuous line of porcine kidney cells (PK) was obtained from Dr. A. J. Knaizeff, Chief Cell Culture Division, Naval Biological Laboratory, Oakland, California. These cell cultures were derived from a culture established at the Cutter Laboratories, Berkeley, California, and cloned in the laboratory of Dr. Morgan Harris at the University of California.

Stock cultures of HeLa and PK cells were grown in 250 ml Kimax bottles at 36 C. When monolayers were complete, the medium was aspirated, replaced with 10 ml of trypsin solution, and incubated at 36 C for 10 minutes. The cells were detached from the glass by gentle pipetting and were packed by centrifugation at 500 x g for five minutes at room temperature. Cells were resuspended in growth medium and counted. Approximately 3×10^6 cells were added to 250 ml bottles and $2\text{-}3 \times 10^5$ cells were used to seed tube or coverslip monolayers. Growth medium was changed every three days and monolayers were usually complete in five to seven days.

Stable lines of cell cultures were periodically treated with Kanamycin[10] (100 mcg/ml) for 10 days to ensure that they remained free from pleuropneumonia-like organisms.

Primary Chick Embryo Cell Cultures

Nine to 11 day-old embryos were harvested and eyes, beaks, wings, and legs were removed and discarded. Torsos were rinsed three times in warm HBSS and minced into three mm pieces with fine scissors. After three additional washes, minced tissues were forced through a 50 ml syringe into a 250 ml fluted trypsinizing flask[11]. Fifty ml of trypsin solution was added to the rinsed tissue and the mixture was stirred with a magnetic stirrer for 15 minutes. The cells were decanted through six layers of sterile gauze into a 250 ml centrifuge tube at room temperature. The process was repeated until a total of four 15-minute trypsinization periods were completed. The cells were centrifuged at 80 x g for 10 minutes to remove the trypsin, resuspended in HBSS, and recentrifuged for 10 minutes. The packed cells were suspended in 50-100 ml of outgrowth medium and counted. Suspensions containing 2-3 X 10^6 viable cells per ml were placed in one-half ml amounts in rubber-stoppered pyrex tubes and incubated at 36 C as stationary cultures in racks[12] inclined at an angle of 10 degrees from the horizontal. Monolayers were usually complete in 48 hours and remained intact for one week when prepared in this manner.

Viable cell counts were made in a counting fluid containing 0.1%

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[10]Kanamycin sulfate, Bristol Laboratories, Syracuse, N. Y.

[11]Bellco Glassware Company, Vineland, N. J.

[12]Seelye Craftsman, Minneapolis, Minn.

trypan blue with 0.1% sodium citrate added to minimize cell clumping. Cells were counted in each corner square and in the central square of each chamber of a hemacytometer[13] (10 square millimeters in all) using 10X objective and ocular lenses (Merchant et al., 1964). Counts were computed by multiplying the number of unstained cells in 10 squares by an appropriate dilution factor.

Production of Viral Pools

Vaccine

Confluent HeLa cell monolayers grown in 250 ml dilution bottles in HLY-10 were inoculated with $10^{5.8}$ $TCID_{50}$ of virus contained in 0.2 ml. Virus was allowed to adsorb for 2-3 hours at room temperature before addition of 10 ml of EMEM-5 per bottle. After 48 hours of incubation at 36 C, the inoculated monolayers were beginning to slough from the glass. Virus was released from the cells by three alternate freeze-thaw cycles at -70 C and 37 C, respectively. The pooled supernatant fluids, removed after centrifugation in the cold at 1000 x g for 15 minutes, were stored at -70 C. The titer of the viral pool used for the production of vaccine was 6.2 log $TCID_{50}$ per ml. Vaccine was prepared by mixing equal parts of this live virus with Freund's complete adjuvant[9].

Clearance Studies

Yolk sacs of embryonated eggs were inoculated with egg-adapted virus containing approximately 1.6 X 10^3 $TCID_{50}$ per 0.5 ml. Embryos dying within 36 hours after inoculation were discarded. After incubation

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[13]Spencer Bright-Line Hemacytometer, American Optical Company, Buffalo, N. Y.

at 36 C for 72 hours when more than 50% of the embryos were dead, allantoic and amniotic fluids were harvested, pooled, and immediately prepared for concentration of the virus. Crude virus contained in the pooled fluids was centrifuged at 1000 x g for 10 minutes to remove extraneous tissues. The supernatant fluid was placed in 30 to 40 ml amounts in sterile polypropylene centrifuge tubes and centrifuged at 22,000 x g for 60 minutes in a Type RC-2 Servall refrigerated centrifuge. The supernatant fluid was decanted and viral pellets were resuspended in one-tenth volumes of either distilled water or HBSS which contained 10% inactivated calf or hamster serum. Suspensions were allowed to stand overnight at 4 C. Pellets were resuspended by gentle pipetting, pooled, and centrifuged at 1200 x g for 15 minutes in the cold. The supernatant fluid from this centrifugation was distributed in convenient amounts in rubber stoppered pyrex tubes. Distilled water preparations were kept at 4 C and used within one week with little loss of titer. Serum preparations were stored at -70 C until used. Titers of viral pools prepared in this manner usually contained $10^{6.5}$ to $10^{7.5}$ $TCID_{50}$ per ml.

Virus Titrations

Samples to be assayed for viral content were thawed rapidly in a 37 C water bath. All tubes and reagents were maintained in an ice bath. Separate one ml pipets were used to prepare each dilution. Serial ten-fold dilutions of stock virus, blood, serum-virus mixtures, or tissue suspensions were prepared in BGS or HLY-10 and inoculated in 0.1 or 0.2 ml amounts in replicate tube monolayers. After adsorption for three at room temperature, monolayers were rinsed twice to remove hemoglobin and tissue debris and 0.5 ml of maintenance medium was added. Monolayers

were incubated at 36 C for one week and observed for the appearance of the characteristic giant cell cytopathic effect (CPE) as described by Gray et al., 1958. Titers were computed according to the method of Reed and Muench (1938) and are expressed as the positive $\log_{10}$ of the dilution producing infection in 50% of the monolayers inoculated with one ml volumes (Log_{10} $TCID_{50}$ per ml). Titers reported for organs are expressed as $TCID_{50}$ per g or per organ.

Preparation of Antiserum

Adult albino rabbits, after preimmunization bleedings, were inoculated intramuscularly at two week intervals for a total of three immunizations (Table 1). Two weeks after the third injection, animals were bled by cardiac puncture and the sterile inactivated serum was checked for neutralizing activity. If low levels of antibody were present, injections were continued until satisfactory neutralizing titers were produced. Animals were bled by cardiac puncture; the resulting sera were inactivated by heating at 56 C for 30 minutes, filter sterilized, and were stored at -25 C before testing.

Neutralization Tests

The constant virus-decreasing serum method of neutralization test was used. Fourfold dilutions of antisera were made in HBSS so that final concentrations of serum when mixed with an equal volume of virus were 1:4, 1:16, 1:64, 1:256, and 1:1024. Virus was diluted to contain 250 $TCID_{50}$ when mixed with diluted antisera. Virus-serum mixtures were incubated for one hour at room temperature before 0.2 ml of each dilution was added to each of five replicate monolayers. After an adsorption

TABLE 1

IMMUNIZATION SCHEDULE FOR PRODUCTION
OF RABBIT ANTIHERPES SERUM

Injection Number	Antigen	Amount	Injection Site	Time Elapsed, Last Injection	Trial Bleedings
1	Virus +[a] adjuvant	1 ml	R. thigh[b]	0	Preinjection
2	Virus +[a] adjuvant	1 ml	L. thigh	2 weeks	
3	Virus +[a] adjuvant	1 ml	R. thigh	2 weeks	6 weeks
4	Virus +[a] adjuvant	1 ml	L. thigh	1 month	
5	Virus +[a] adjuvant	1 ml	R. thigh	1 month	
TOTAL IMMUNIZATION PERIOD					4 months

[a]Equal parts of live virus (6.2 log $TCID_{50}$ per ml) plus Freund's complete adjuvant.

[b]Intramuscular injection.

period of three hours, monolayers were rinsed twice to remove residual serum and maintenance medium was added. Monolayers were incubated at 36 C and observed for one week for characteristic CPE. Neutralization indices were computed as described by Lennette, (1964).

Preparation of Fluorescein Labeled Globulin

Fractionation of Serum Proteins

Both rabbit antiherpes and normal rabbit sera were fractionated. Twenty ml of serum were added to an equal volume of cold distilled water in an 125 ml erlenmeyer flask containing a magnetic stirring bar. The mixture was agitated slowly in an ice bath during the dropwise addition of 40 ml of 4.1 M ammonium sulfate from a separatory funnel. The first precipitate was allowed to stand at 4 C overnight, followed by centrifugation at 1000 x g for 30 minutes to pack the globulin. The supernatant fluid was discarded and the globulin was dissolved in measured amounts of cold distilled water. The globulin was precipitated and redissolved two additional times using amounts of saturated ammonium sulfate equal to the volumes of water necessary to dissolve the globulin. The final precipitate was dialyzed in 0.005 M phosphate buffered saline, pH 7.2, until sulfate was no longer detectable in the dialysate as shown by the addition of saturated barium chloride.

Protein Determination

Protein concentration of the dialyzed globulin was determined using the biuret reaction of Gornall *et al.* (1949). Protein standards[14]

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[14]Crystalline bovine albumin, Armour Pharmaceutical Company, Kankakee, Ill.

were prepared to contain 0.5, 1.0, 1.5, 2.0, and 2.5 mg protein per ml. Unknown globulins were diluted 1:10 and 1:20 in distilled water. To one ml portions of test solutions (standards, unknowns, and distilled water) in cuvettes, four ml of biuret reagent were added and allowed to stand at room temperature for 30 minutes. Optical density was read at 540 mμ using a Coleman junior spectrophotometer, and the concentration of protein in the globulin was determined from a standard curve for known protein.

Labeling of Globulins

The globulin was diluted to contain 10 mg of protein per ml in carbonate-bicarbonate buffer, pH 9, and saline. The mixture was chilled to 0-4 C, and fluorescein isothiocyanate[15] was added at a ratio of 0.05 mg for each mg of protein in solution. The mixture was placed in the refrigerator and stirred with a magnetic stirrer for 18 hours.

Removal of Unbound Fluorochrome

The conjugate was dialyzed overnight in a liter of 0.005 M phosphate buffered saline, pH 7.2, containing 10 grams of Dowex[16]. The buffer was discarded, fresh buffer without Dowex added, and dialysis was continued until no dye appeared in the dialysate. The dialyzed conjugate was passed through an amberlite[17] column prepared by washing with 3 N

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[15]Isomer 1, crystalline M. A., Mann Research Laboratories, New York, N. Y.

[16]Dowex 2-X4, chloride form, 20-50 mesh, J. T. Baker Company, Phillipsburg, N. J.

[17]Amberlite, C G 400, chromatographic grade, 100-200 mesh, Mallinckrodt Chemical Works, St. Louis, Mo.

HCl and rinsing in phosphate buffer until the pH of the column was restored to 7.2 to 7.5.

Absorption of Nonspecific Protein

Conjugates were absorbed prior to use with hamster liver powder prepared commercially[18] as described by Coons et al. (1955). Powder washed in phosphate buffered saline was added at a concentration of 100 mg per ml of conjugate. After absorbing for one hour at room temperature, absorbant was removed by centrifugation at 10,000 x g for 10 minutes. A second treatment using rabbit bone marrow powder[19] was similarly performed. Aqueous merthiolate[20] was added to the absorbed conjugate at a concentration of 1:10,000, and one ml portions were placed in stoppered pyrex tubes and stored at -25 C.

Titration of Labeled Globulin

Twofold dilutions of the labeled globulin were prepared in phosphate buffered saline. Frozen sections of infected tissues were air dried, fixed in acetone for 5 to 10 minutes, and allowed to air dry. The various dilutions of conjugate were added to sections and incubated in a moist atmosphere for 30 minutes at room temperature. Slides were rinsed twice in buffered saline and coverslips were mounted over sections in a drop of buffered glycerol saline. Slides were examined with a Reichert fluorescence microscope equipped with a darkfield condenser. The light source was a mercury HBO 200 burner using Corning 5970 or 5840 exciter

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[18]Pel-Freez Biologicals, Inc., Rogers, Ark.

[19]Baltimore Biologicals Company, Baltimore, Md.

[20]Eli Lily and Company, Indianapolis, Ind.

filters and a Wratten 2A barrier filter. The highest dilution of conjugate producing maximum specific fluorescence and minimum background staining was selected for experimental use.

Clearance Studies

Adult male hamsters with an average weight of 130 grams were anesthetized by intraperitoneal injections of sodium pentobarbital[21] in doses recommended by Whitney (1963). Intravenous injections of one ml volumes of virus were made in the exposed left jugular vein. Blood samples were removed from alternate jugular veins or from the heart using tuberculin syringes with one-half inch, 26 gauge disposable needles. They were mixed with equal volumes of HLY-10 with added 2% sodium citrate and frozen at -70 C. Samples were taken before virus injection, within one minute after injection, and at five or 10 minute intervals thereafter for 90 minutes. Preinoculation samples of blood which were added to equal volumes of diluent and virus, served as the input virus control for each animal. Titers of samples removed one minute after viral injection were plotted as circulating virus titers at time zero. Average titers observed for these samples agreed within 0.3 $\log_{10}$ $TCID_{50}$ of theoretical titers adjusted for blood dilution, when blood volumes were calculated as 8% of the total body weight of an animal. Organs were removed at varying time intervals after viral injection. Gallbladders and extraneous tissues were removed, and organs were rinsed in HBSS and frozen at -70 C. Organs were thawed at 37 C and ground in a TenBroeck tissue homogenizer. A 20% weight-to-volume suspension was made in HLY-10 for assay of viral

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[21]Diabutal, Diamond Laboratories, Des Moines, Iowa

activity. Concentration of virus in organs compared to that present in the circulating blood at the time of organ removal was estimated using organ blood volume measurements for normal rats as reported by Lewis et al. (1952). Concentration factors were obtained by dividing the actual titers of organs by the titers estimated to be due to organ blood content.

Serum Inhibition Studies

Blood was obtained from anesthetized adult male hamsters by cardiac puncture, allowed to clot at room temperature, and kept at 4 C overnight. Pooled sera (NHS) were centrifuged to remove blood cells and stored at -70 C until used. Inactivated hamster serum (IHS) was prepared by heating NHS at 56 C for 30 minutes. Serum deficient in properdin (RP) was prepared by a modification of Pillemer's method as follows (Barlow et al., 1958). Zymosan[22] was suspended in sterile 0.85% saline so that three mg were contained in 0.1 ml. Both serum and zymosan suspensions were brought to 16 C in a constant temperature water bath and 0.2 ml of the zymosan suspension was added to two ml of serum which was mixed, and maintained at 16±1 C for one hour. Samples were shaken at 10 minute intervals. Suspensions were centrifuged at 1200 x g for 15 minutes in the cold, the supernatant fluid decanted, and the procedure was repeated. The clear supernatant fluid was stored at -70 C until used. Heat inactivated serum which served as a zymosan control (RPI) was similarly prepared. Assays for serum inhibition were made with serial tenfold dilutions of virus, 1:10 diluted virus, and undiluted virus. These were incubated

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[22]Lot #5818, Immunological grade, Nutritional Biochemical Corp., Cleveland, Ohio.

with equal volumes of serum at 35 C for varying time periods. Serum samples mixed with undiluted or tenfold diluted virus were serially diluted in HLY-10 and assayed in replicate tube monolayers after the various incubation periods.

Blockade of the Reticuloendothelial System

Blockade of the reticuloendothelial system was accomplished with an ethyl stearate emulsion (Cooper and Stuart, 1961; Stuart and Cooper, 1962; Cooper and Stuart, 1962). One gram of ethyl stearate[23] was added to four ml of dextrose water containing 0.04 ml of Tween 20[24] and heated to 50 C. The suspension was homogenized for five minutes at high speed in a Servall Omnimixer[25] and kept at 40 C until used. Animals were injected intracardially 24 hours prior to clearance studies with either the lipid emulsion or pyrogen-free saline[26]. The blocking dose administered was one mg of ethyl stearate per gram of total body weight of the animal.

Assay of Blood Components

Blood was withdrawn from the hamster heart 30 to 60 minutes after intravenous inoculation of virus. It was mixed with an equal volume of 2% citrate-saline solution and centrifuged at 550 x g for 10 minutes in a refrigerated centrifuge. Citrated plasma was removed from the packed cells and centrifuged at 2000 x g for 30 minutes to bring down the

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[23]Eastman Organic Chemicals, Rochester, N. Y.

[24]Polyoxyethylene sorbitan monolaurate, Sigma Chemical Company, St. Louis, Mo.

[25]Ivan Sorvall, Inc., Norwalk, Conn.

[26]Cutter Laboratories, Berkeley, Calif.

platelets. Platelets were washed twice with 10 ml volumes of HBSS after which they were resuspended for titration in a volume of HLY-10 equal to the original volume of blood withdrawn. Cells were washed five times in 10 ml volumes of HBSS and were also brought back to initial blood concentration in HLY-10. Samples of undiluted blood, cell rinses, and blood cells were assayed for viral content.

Studies on the Fate of Injected Virus in Organs

Groups of hamsters were anesthetized and inoculated with one ml of virus 10^7 $TCID_{50}$ intracardially. Animals were sacrificed at varying time intervals for three days, samples of blood taken, and organs removed and washed in HBSS.

Preparation of Tissue Sections

Tissues for fluorescent antibody staining were placed in aluminum foil, frozen on dry ice blocks, and stored at -25 C. Sections (4 to 6 microns in thickness) were cut in a cryostat at -25 C. Sections on glass slides were dried at room temperature for one hour, fixed in acetone for 10 minutes, and dried at room temperature for 10 minutes. Fluorescent antibody staining was done by both the direct and indirect methods.

Direct method. Fixed sections were treated with fluorescein labeled rabbit antiherpes globulin for 30 minutes at room temperature in a moist atmosphere to prevent drying. Slides were washed in two changes of phosphate buffered saline, pH 7.5, 10 minutes each wash. One part of rhodamine-conjugated bovine albumin[19] was added to 20 parts of fluorescein conjugate as a counterstain to minimize nonspecific fluorescence. After two additional washes for 10 minutes in buffered saline, sections were

mounted in buffered glycerol saline and examined with a Reichert microscope equipped with a cardioid darkfield condenser and appropriate filters. The light source was an Osram HBO 200 high pressure mercury lamp. Specificity of staining was determined by staining normal tissues with specific conjugate; staining infected tissues with normal rabbit conjugate; and, inhibition of specific staining in infected tissues by pretreatment with immune rabbit serum.

Indirect method. Acetone-fixed tissue sections were treated with pooled human-antiherpes serum for 30 minutes, washed twice in buffered saline for 20 minutes, and stained with rabbit anti-human globulin (Weller and Coons, 1954) for 30 minutes. Both sera were absorbed with hamster liver powder, and rhodamine-conjugated bovine albumin was added to the fluorescein-conjugated globulin as a counterstain. Microscopic examination was similar to that employed with the direct method.

Photomicrographs of preparations stained by both the direct and indirect methods were taken with a 35 mm Leica camera fitted with a Micro-Ibso attachment[27]. Both Kodacolor-X and Kodak Tri-X Pan films[28] were used.

Conventional histological staining of tissue with hematoxylin and eosin was performed on formalin fixed tissues and on frozen sections.

Titrations for Viral Content

Blood and organ samples were titrated for viral content as previously described except that parts of organs were used instead of whole organs.

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[27]E. Leitz, Inc., New York, N. Y.

[28]Eastman Kodak Co., Rochester, N. Y.

CHAPTER IV

EXPERIMENTAL RESULTS

Effect of Anticoagulants Upon Herpesvirus

It was necessary to use an anticoagulant in the collection of blood samples in order to prevent clotting prior to viral titration. Since heparin was known to inhibit the virus used in these studies (Nahmias and Kibrick, 1964), the effect of other anticoagulant substances upon the virus was investigated. Comparative titrations of HSV were made in the presence of two other anticoagulants. Representative results of these experiments are presented in Table 2. When compared with the control titration in buffered gelatin saline, no inhibition of viral infectivity occurred when solutions containing virus and 2% sodium citrate were incubated in an ice bath for two hours prior to assay. Similar incubation of the virus in a 1% solution of the disodium salt of ethylene diamine tetra-acetic acid (EDTA) resulted in a 69% loss of viral activity. As a result of these findings, diluents containing 2% sodium citrate were used subsequently in all blood collecting procedures.

Production of Antiherpes Serum in Rabbits

Neutralizing titers of antisera produced in four rabbits are presented in Table 3. None of the animals had demonstrable neutralizing antibody titers prior to immunization. Low, but detectable, antibody titers

TABLE 2

EFFECT OF ANTICOAGULANTS ON VIRAL INFECTIVITY

Concentration of Anticoagulant	Viral Titer[a]	Percent Inhibition
Sodium citrate, 2%	6.4	0
EDTA, 1%	5.7	69
BGS[b] + virus (Control)	6.2	

[a]Log_{10} $TCID_{50}$ per ml.

[b]Buffered gelatin-saline.

TABLE 3

NEUTRALIZING ACTIVITY OF ANTIHERPES SERUM AND YIELD PER RABBIT

Animal Identity	Serum Samples			Final Yield (ml)
	Preimmunization	Post-immunization		
		Six Weeks	Four Months	
A	NA[a]	1:56[b]	1:500	55
B	NA	1:16	1:110	35
C	NA	1:11	1:110	18.5
D	NA	1:11	1:28	43

[a]No activity.

[b]Dilution of serum which neutralized 250 $TCID_{50}$ of HSV.

were observed in all animals six weeks after immunization was begun. Serum from animal A contained a fivefold greater activity than did that of other animals. Five injections and approximately four months after beginning the immunization series, titers were higher for all animals. Approximately tenfold increases in neutralizing activity were shown for all animals except D, and the titer of this animal's serum was two and one-half times greater than that obtained in previous tests. One animal (A) produced an antiserum with a titer approximately five times greater than that of any other animal. Yields of serum are listed in Table 3; volumes ranged from a high of 55 ml to 18.5 ml. Only 50 ml of blood was removed from the animal yielding the lowest volume of serum.

Titration of Fluorescein-labeled Globulin

Results of titration of fluorescein-labeled antiherpes globulin prepared from serum obtained from animal A are shown in Table 4. Activity of the conjugated globulin was relatively low, since maximum fluorescence was not present in viral-infected sections stained with dilutions greater than 1:4. Specificity of the observed reactions was indicated by lack of specific fluorescence in uninfected tissue sections and in infected tissues treated with specific antiserum.

Concentration of Virus for Clearance Studies

Results of concentration procedures of four representative viral pools are shown in Table 5. Final infectivity titers of concentrated and partially purified virus were from three to eight times greater than titers obtained for the original pooled allantoic and amniotic fluids. In addition to achieving greater concentration of viral particles per

TABLE 4

TITRATION OF FLUORESCEIN-LABELED ANTIHERPES GLOBULIN[a]

Preparation	Dilutions			
	0	1:2	1:4	1:8
Tissue sections, infected with herpesvirus	+[b]	+	+	±
Tissue sections, uninfected	±	±	0[d]	0
Tissue sections, infected with herpesvirus, pretreated with specific antiserum	0	0	0	0

[a]Globulin absorbed with hamster liver and rabbit bone marrow powders. It also contained 5% rhodamine-albumin.

[b]Maximum yellow-green fluorescence observed, infected cells sharply defined.

[c]Some yellow fluorescence noted, cells not sharply defined.

[d]No definite specific fluorescence observed; blue autofluorescence seen.

TABLE 5

CONCENTRATION OF HERPESVIRUS BY CENTRIFUGATION[a]

Pool Number	Crude Virus[a]		Concentrated Virus[b]		Concentration
	Titer[b]	Volume (ml)	Titer[c]	Volume (ml)	
1	6.0	200	6.7	20	5x
2	6.5	320	7.0	32	3.2x
3	6.2	420	7.0	42	6.3x
4	6.6	240	7.5	24	8x

[a]Pooled allantoic and amniotic fluids from infected embryonated eggs.

[b]Viral pellet resuspended in HBSS + 10% calf serum, one tenth original volume.

[c]Titers expressed as positive $\log_{10}$ $TCID_{50}$ per ml.

volume of diluent, concentration procedures also permitted the removal of egg fluids and debris from the viral pellet and resuspension in the desired medium.

Sensitivity of Cell Cultures to Virus

Results obtained from comparative titrations of virus in HeLa and primary chick embryo (CE) cell cultures led to the adoption of the latter for the assay of virus present in blood and tissues. Titers were consistently 10 times higher in CE cell cultures than in HeLa cells. The results of a comparative titration are presented in Table 6.

Stability of Viral Pools in Storage

The preparation of high titered viral pools for use in long term experiments requires that little loss of activity occurs in storage. Since it was necessary to use virus suspended in distilled water in certain phases of this investigation, studies to determine the stability of HSV suspended in water as compared to preparations in HBSS and 10% serum were performed. A decline in titer of 0.2 $\log_{10}$ $TCID_{50}$ occurred with virus suspended in distilled water when it was stored at 4 C for one week (Table 7). Virus suspended in water and stored in this manner was always used within one week after preparation. Titers of virus suspended in serum and stored at -70 C declined 0.5 $\log_{10}$ $TCID_{50}$ after 18 days. Samples tested as long as 75 days after preparation showed no loss in titer from that observed at approximately three weeks.

Effect of Serum on Viral Clearance

Since it was possible that serum used to stabilize the virus during long term storage at -70 C might influence clearance rates in

TABLE 6

SENSITIVITY OF TWO CELL CULTURES TO HERPESVIRUS

Culture	Log_{10} $TCID_{50}$ per ml
Primary chick fibroblasts	7.2
HeLa	6.2

TABLE 7

STABILITY OF STORED VIRAL POOLS

Days After Preparation	Viral Titer[a]	
	Water[b]	Serum[c]
0[d]	7.4	7.5
4	7.2	---
7	7.2	---
18	---	7.0
75	---	7.0

[a]Log_{10} $TCID_{50}$ per ml.

[b]Suspended in distilled water and stored at 4 C.

[c]Suspended in 10% calf serum and stored at -70 C.

[d]Original titer.

intravenously-injected hamsters, viral pools were prepared in homologous and heterologous (calf) sera as well as in distilled water to determine the effect of the suspending medium upon clearance of virus. Figure 1 summarizes results of experiments in which titers were obtained for residual virus in the blood at varying time intervals after intravenous injection of three viral preparations. Titers for each interval are average values obtained from four animals for each viral suspension. In all preparations 50% of the virus disappeared five minutes after inoculation. Times by which half of the injected virus had disappeared (T/2) were five minutes for the distilled water preparation, and three minutes for both serum preparations. In 10 minutes 90% of the circulating virus was not detectable in any preparation. Individual times of 90% disappearance observed for distilled water, hamster serum, and calf serum suspensions were eight, nine, and seven minutes, respectively. At 30 minutes, 99% of the virus had disappeared, although low levels of virus were detected in the circulating blood for the duration of the experiments (90 minutes). No significant difference in disappearance rates were noted for virus suspended in distilled water or in either of the inactivated serum preparations. Results of these experiments indicated that calf serum could be used as a stabilizing agent in viral pools without influencing clearance rates, and all subsequent experiments were performed using virus prepared in this manner.

Clearance of Herpesvirus from Hamster Blood

A typical curve showing the disappearance of virus from the circulating blood of the hamster is shown in Figure 2. Each point on the curve represents the average blood titer for four animals and vertical

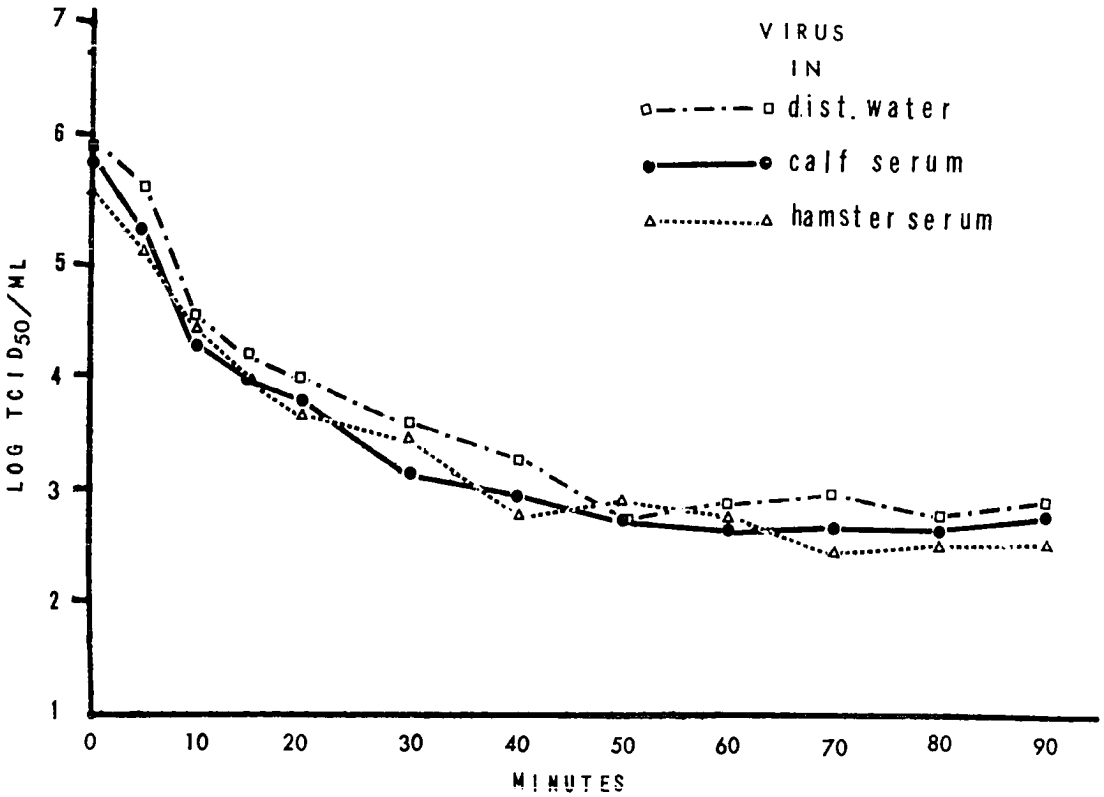

Figure 1. Effect of serum on disappearance of herpesvirus from hamster blood.

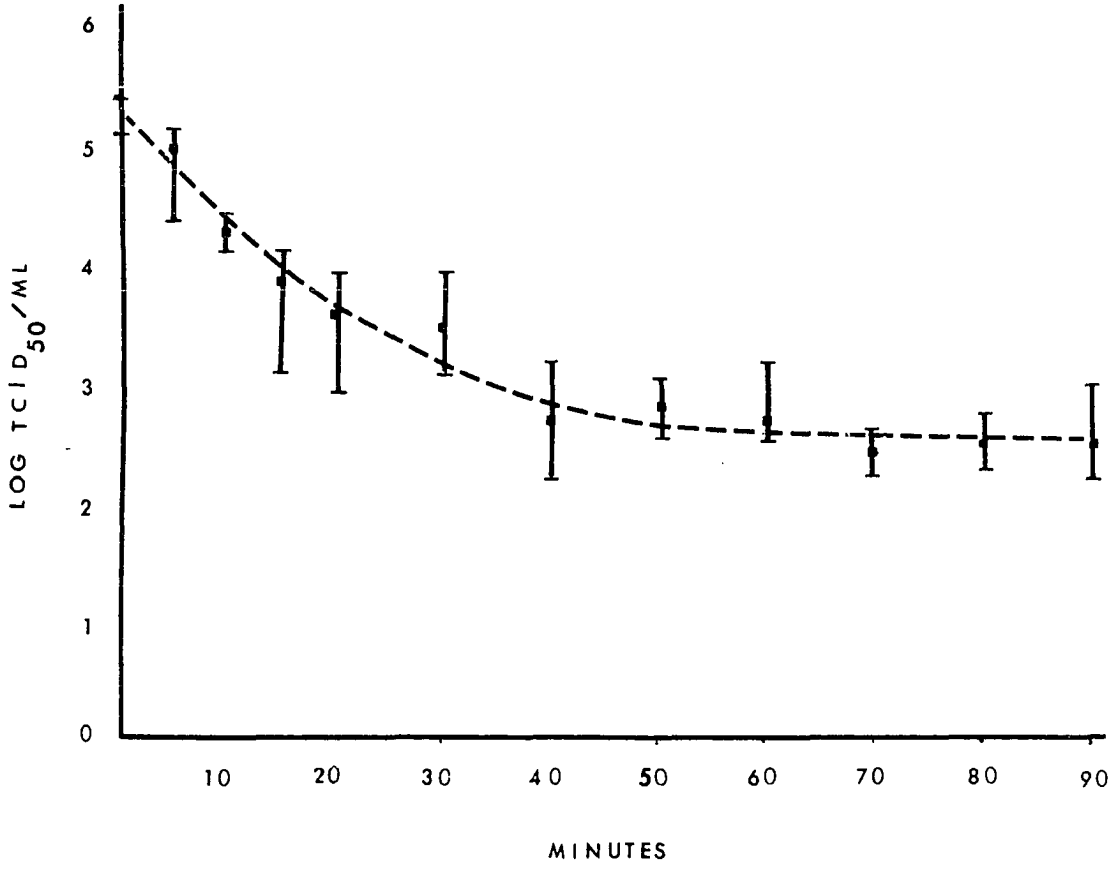

Figure 2. Rate of clearance of herpesvirus from hamster blood.

bars represent the range of titers obtained for each time interval. Half times (T/2) of viral disappearance always occurred within five minutes after inoculation. By 10 minutes, 90% of the virus was no longer detectable, and at 30 minutes 99% of the virus had disappeared. After the initial rapid clearance of most of the virus, low, but detectable, levels (500 $TCID_{50}$) of virus persisted in the circulating blood for at least 90 minutes.

The possible effect of smaller quantities of virus upon clearance was tested by injecting a 1:100 dilution of the usual virus dose (10^7 $TCID_{50}$). The characteristic pattern of clearance obtained with large doses was also observed with the smaller inoculum (Figure 3). Half of the injected virus disappeared within three minutes and 90% and 99% of the virus was no longer detectable in the blood at five and 20 minutes, respectively. Although only 99% of the usual virus dose was injected, an uncleared fraction of virus (32 $TCID_{50}$) was present in the circulating blood for one hour. The titer of the persisting viral fraction, however, was lower than that found in animals injected with the larger inoculum.

Fractions of Hamster Blood Containing Virus

Experiments were performed to determine if the small amount of virus persisting in the circulating blood might be associated with certain formed blood elements. Blood removed from animals one hour after injection of virus was placed in 2% citrate-saline and cells were separated from plasma by centrifugation. Approximately 73% of the viral activity present in whole blood one hour after injection was recovered in various blood components after centrifugation and washing procedures (Table 8). Most of this activity (62.5%) was in the plasma fraction.

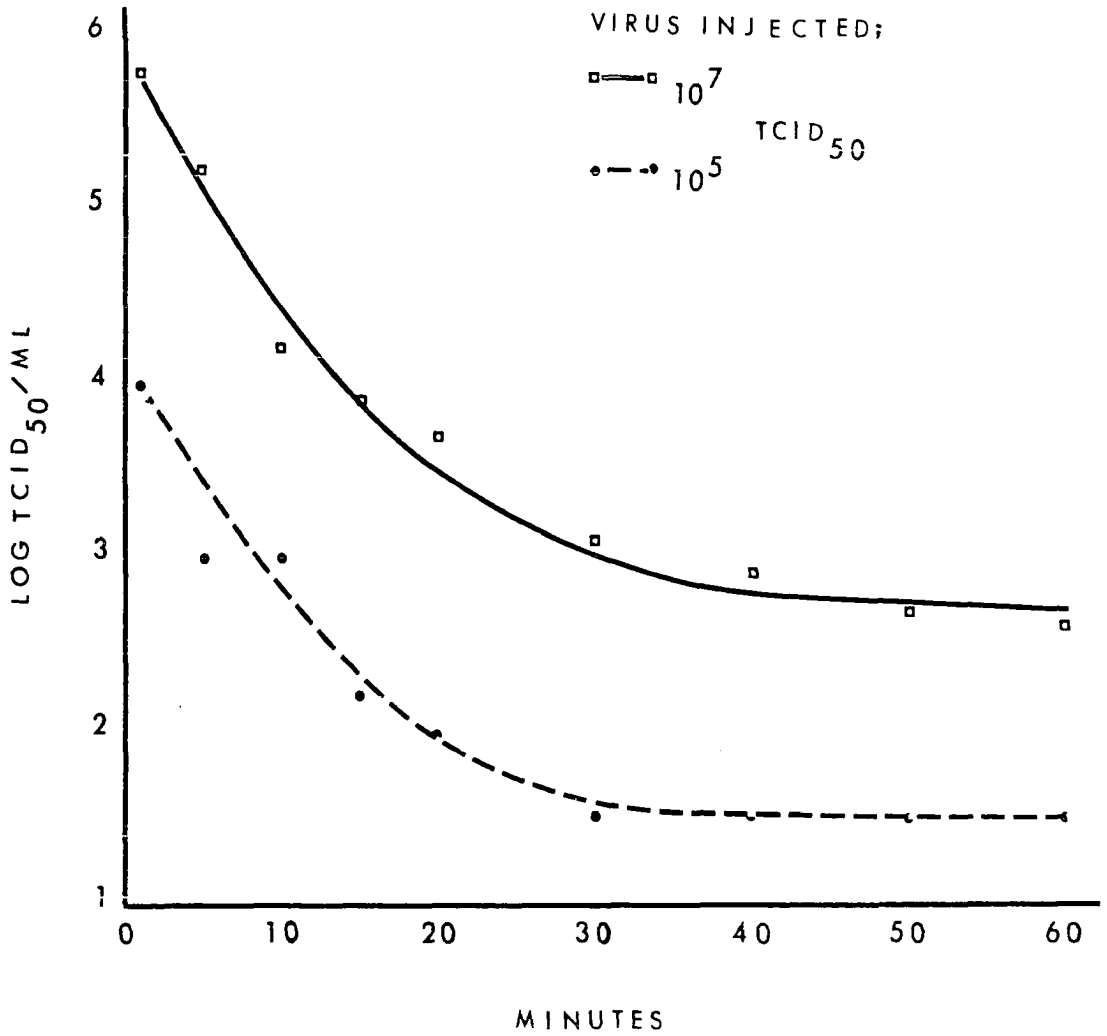

Figure 3. Effect of dose of herpesvirus on blood clearance rate.

TABLE 8

VIRUS IN HAMSTER BLOOD FRACTIONS ONE HOUR AFTER INTRAVENOUS INOCULATION

Blood Fraction	$TCID_{50}/ml$	Percentage[a]
Whole blood	1600	100
Plasma, 550 x g	1000	62.5
Plasma, 2000 x g	1000	62.5
Washed platelets	< 1	0
Washed RBC & WBC	160	10
Total recovered	1160	72.5

[a]Percentage of virus obtained from blood fractions.

The fraction containing red and white cells, which had been washed five times in 10 volumes of HBSS, contained 10% of the viral activity. No activity was associated with washed platelets.

Recovery of Virus in Hamster Organs

Although 99.9% of the injected virus disappeared from the circulating blood at 90 minutes, titration of liver and spleen homogenates revealed that only negligible amounts could be recovered from these organs (Table 9). Total amounts of virus recovered from blood, liver, and spleen at this time were less than 4% of the injected virus. The degree of concentration of virus in the organs as compared to that of the circulating blood at the time of organ removal, was estimated according to the organ blood volume measurements reported by Lewis *et al.* (1952) for normal rats. With these values, the expected concentration of virus within an organ due to blood volume alone could be determined. The concentration factor was computed by dividing the actual titer of the organ by the calculated titer due to organ blood volume. Concentration factors for three viral preparations ranged from 210 to 260 in the spleen and from 280 to 710 in the liver (Table 9).

Since the observed low level of recoverable virus possibly could be due to adsorption to cells of organs of the RES with subsequent entry into the eclipse phase, organs removed 10 minutes after virus inoculation were assayed for viral content. Lungs, brain, heart, adrenals, and kidneys were examined in addition to the spleen and liver. As shown in Table 10, 57% of the injected virus was recovered in the organs examined. Organs other than circulating blood which contained significant amounts of virus were the liver (16%), spleen (0.8%), and the lungs (22%). The

TABLE 9

VIRUS IN HAMSTER ORGANS NINETY MINUTES AFTER INJECTION

Viral Preparation	$TCID_{50}$ per Organ			Virus			Concentration Factor	
	Liver	Spleen	Blood	Input[a]	Recovered[a]	Percentage Recovered	Liver	Spleen
Distilled water	240,000	11,000	7,600	10,000,000	260,000	2.6	450	260
Hamster serum, 10%	91,000	5,800	3,800	4,000,000	100,000	2.5	280	210
Calf serum, 10%	290,000	8,200	5,500	7,900,000	300,000	3.8	710	260

[a]Expressed as $TCID_{50}$

TABLE 10

VIRUS IN HAMSTER ORGANS TEN MINUTES AFTER INJECTION

Organ		Recoverable Virus		
Identity	Weight (g)	$TCID_{50}$ per gram	$TCID_{50}$ per organ	Percentage[a]
Liver	4.85	32,000	160,000	16.0
Spleen	0.25	32,000	8,000	0.8
Adrenals	0.05	1,600	80	0.008
Kidneys	1.05	3,200	3,400	0.34
Lungs	0.7	320,000	220,000	22.0
Heart	0.5	3,200	1,600	0.16
Brain	0.65	100	65	0.0065
Blood	11 ml	16,000	180,000	18.
Totals			570,000	57.0

[a]Obtained by dividing $TCID_{50}$ per organ by input virus. Input virus was 1×10^6 $TCID_{50}$.

kidney, heart, brain and adrenals contained a lower percentage of the total virus. Estimated concentration factors in organs for which blood volume estimates were available are presented in Table 11. The degree of concentration of virus in the liver (11.4) and spleen (4.2) was considerably lower at 10 minutes than at 90 minutes, although percent recovery per organ was greater at 10 minutes than at 90 minutes. A high concentration factor of 32 was observed to occur in the lungs which was 3 to 8 times greater than that found in the liver and spleen. Little uptake of virus occurred in the kidneys as indicated by a concentration factor of 0.72.

Recovery of virus in liver and lungs at various time intervals after intravenous inoculation are presented in Figure 4. A single entry is listed for both heart and lungs since organ blood volume estimates used in computing concentration factors were reported for combined organs. Titrations of both organs separately indicated that combined titers represent virus present in the lungs almost exclusively, since heart tissue did not take up virus to an appreciable extent. Computations are based on average values obtained from at least two animals for each time period. At five minutes, 75% of the injected virus was recovered in the organs examined. The liver contained 58% of the input virus, whereas only 0.6% was present in the lungs. The circulating blood contained 18% of the injected virus at five minutes. A marked decline in virus recoverable in organs was observed over the one and one-half hour period of each experiment. Most of the recovered virus was present in the liver at each time interval. Associated with the decline in percent of recoverable virus in organs, as illustrated in Figure 4, was a concurrent increase in amount

TABLE 11

UPTAKE OF VIRUS IN HAMSTER ORGANS TEN MINUTES POST-INOCULATION

Organ				$TCID_{50}$ per Organ		Concentration Factor[c]
Identity	Weight (g)	$TCID_{50}$ per gram	Blood vol. (ml) per gram of organ[a]	Calculated[b]	Actual	
Liver	4.85	32,000	0.178	14,000	160,000	11.4
Spleen	0.25	32,000	0.481	1,900	8,000	4.2
Kidneys	1.05	3,200	0.278	4,700	3,400	0.72
Heart & Lungs (combined)	1.20	323,000	0.379	7,300	230,000	32.0

[a]Values for rat were used (Lewis et al., 1952).

[b]Calculated titer equals organ weight (g) x blood volume per gram of organ x blood titer at 10 minutes.

[c]Concentration factor equals actual titer of organ ÷ calculated titer.

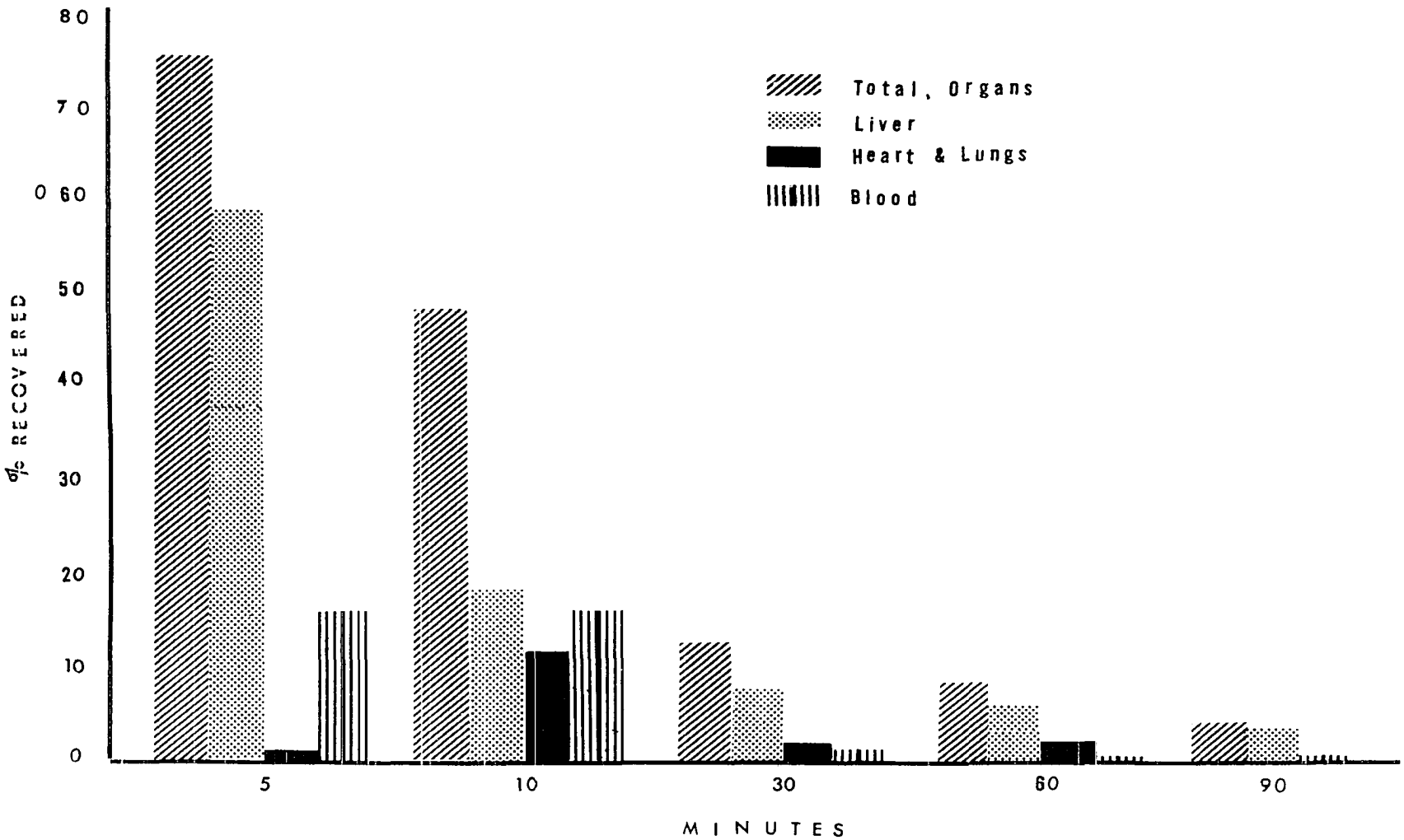

Figure 4. Recovery of herpesvirus from hamster organs at varying times after inoculation.

of virus within organs for the same time interval (Table 12). Uptake of virus in the liver occurred more rapidly than in the spleen and lungs; degree of concentration in the liver was 20 times greater at 90 minutes than the amount recovered at five minutes. Little virus was concentrated in either spleen or lungs at five minutes, but significant amounts occurred in these organs later. Concentration factors for the lungs were not available for the 90 minute-time-interval.

Serum Inhibition Studies

Finkelstein et al. (1959) reported the inactivation of eight strains of herpesvirus by an inhibitor, which they identified as properdin, present in unheated rat serum. Since this agent might play a role in the disappearance of intravenously injected virus, in vitro experiments were performed to determine if this type of inhibition was active in normal hamster serum. Time and temperature conditions approximating those occurring in clearance studies in the living animal were used. A preliminary experiment using heated and unheated pooled hamster serum showed that 98.4% (1.8 $\log_{10}$ $TCID_{50}$) of viral activity was lost in 90 minutes in the presence of normal hamster serum. No loss of activity occurred in heated serum nor in the buffered gelatin-saline control (Table 13). This experiment was repeated with pooled sera from a different group of animals using both heated serum and zymosan treated serum as controls (Table 14). Zymosan absorption of serum at 16 $\pm$ 1 C is reported to remove properdin selectively (Pillemer et al., 1956). An inhibition index of 1.3 $\log_{10}$ $TCID_{50}$ representing a loss of 95% of viral activity was obtained with normal hamster serum at 90 minutes. Small losses in viral activity (0.3 $\log_{10}$ $TCID_{50}$) were noted in both the virus control (HLY-10)

TABLE 12

UPTAKE OF VIRUS IN HAMSTER ORGANS AT VARIOUS TIMES AFTER INTRAVENOUS INOCULATION

Organ	Concentration factor[a]		
	5 minutes	30 minutes	90 minutes
Liver	35.0	310	710
Spleen	0.63	110	260
Heart & Lungs	0.83	66	NR[b]

[a]Concentration factor: Actual organ titer/calculated organ titer

Calculated titer: Organ weight (g.) x blood vol./g. of organ x blood titer at time organ was removed.

[b]Heart and lungs not titrated at this time interval.

TABLE 13

VIRAL INHIBITION IN POOLED NORMAL HAMSTER SERUM

Viral Preparation[a]	Log $TCID_{50}$ per ml	Inhibition[b] index
BGS[c] (control)	6.2	---
Heated Serum	6.2	0.0
Unheated Serum	4.4	1.8

[a]Incubated at 35 C, 90 minutes.

[b]Inhibition index is the difference in Log_{10} $TCID_{50}$ between control and test preparations.

[c]Buffered gelatin saline.

TABLE 14

VIRAL INHIBITION IN POOLED TREATED AND UNTREATED NORMAL HAMSTER SERUM[a]

Viral Preparation	Log $TCID_{50}$ per ml	Inhibition[b] index
HLY-10[c], time zero	6.8	---
HLY-10, 90 min.	6.5	0.3
Normal hamster serum (NHS)	5.5	1.3
Zymosan treated serum, (RP)	6.8	0.0
Zymosan treated, heated serum (RPI)	6.5	0.3

[a]Incubated at 35 C, 90 minutes.

[b]Inhibition index is the difference in Log_{10} $TCID_{50}$ between control and test preparation.

[c]Lactalbumin-yeast extract medium in HBSS and 10% inactivated calf serum.

and the zymosan control (RPI) at 90 minutes.

In order to determine the effect of time of incubation on viral inhibition with the same pooled normal serum, an experiment was designed in which undiluted virus was mixed with several serum preparations and incubated for varying lengths of time before titration (Table 15). No inhibition of virus infectivity occurred in the normal hamster serum under the conditions of the experiment. Since inhibition occurred when normal serum was mixed with tenfold serial dilutions of virus before incubation (Table 14), and did not occur when undiluted virus was mixed with serum (Table 15), additional studies on the effect of viral dilution upon serum inhibition were made. Results of these studies are presented in Figure 5. When virus, in serial tenfold dilutions, was mixed with serum, incubated for the indicated time periods, and titrated, a progressive increase in the inhibition index was observed. The inhibition index increased from 0.8 $\log_{10}$ $TCID_{50}$ at 10 minutes to 1.5 $\log_{10}$ $TCID_{50}$ at 90 minutes. When virus was diluted 1:10 in serum (the approximate dilution which results in vivo with intravenous injection) before incubation, inhibition was maximum at 10 minutes (0.5 $\log_{10}$ $TCID_{50}$), and remained at this level throughout the 90 minute experimental period. Again, no inhibition was observed when undiluted virus was mixed with serum before incubation.

Effect of Blockade of the RES on Viral Clearance

If the disappearance of virus observed in clearance studies was due to uptake by macrophages lining the endothelium of organs of the RES, blockade of the phagocytic function of these cells should not only decrease clearance of virus from the blood, but should also affect the

TABLE 15

LACK OF INHIBITION OF HERPESVIRUS IN TREATED AND UNTREATED NORMAL HAMSTER SERUM USING UNDILUTED VIRUS

Reaction mixture[a]	Incubation time, minutes			
	10	30	60	90
Virus, buffered gelatin-saline (BGS)[b]	5.5[c]	5.5	6.2	5.5
Virus, Normal hamster serum (NHS)	6.0	5.2	6.0	5.5
Virus, heater hamster serum (IHS)	6.0	6.0	5.5	6.0
Virus, zymosan treated serum (RP)	6.0	6.0	6.0	5.5
Virus, heated and zymosan treated serum (RPI)	5.5	6.2	6.2	6.0

[a]Incubated at 35 C.

[b]Titer of virus plus BGS at time zero (virus control), 5.5 log $TCID_{50}$/ml.

[c]Titer expressed as Log_{10} $TCID_{50}$ per ml.

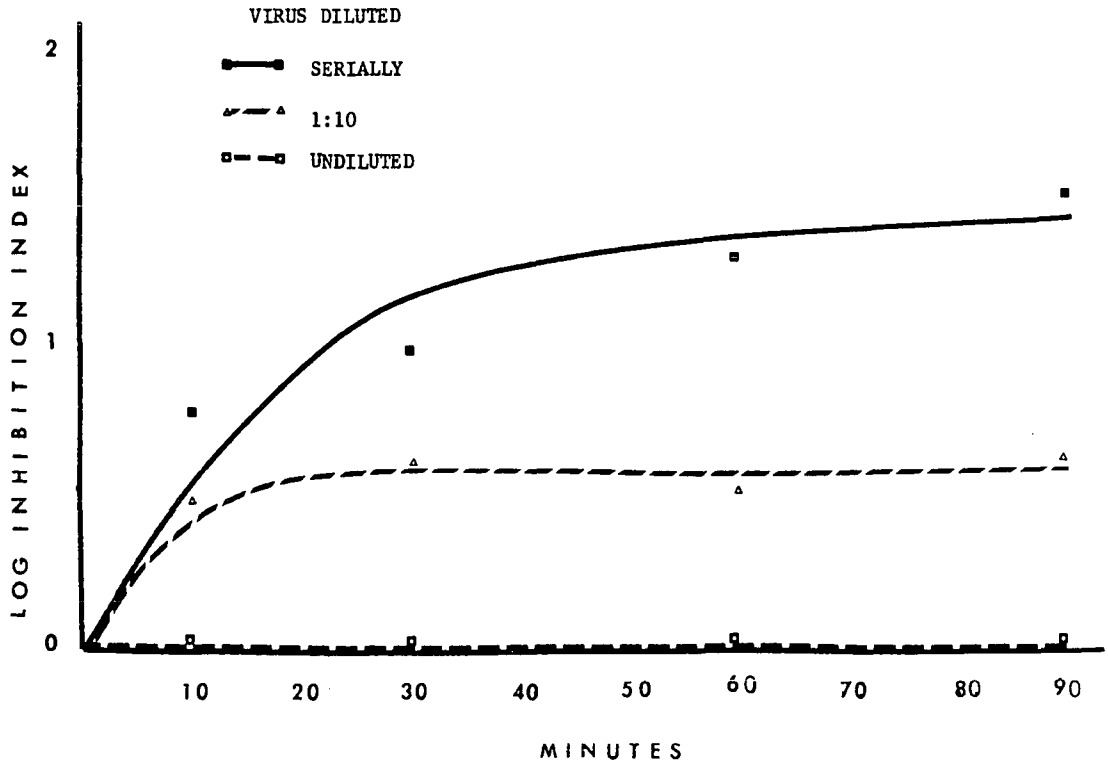

Figure 5. Inhibitory effect of normal hamster serum on infectivity with diluted virus.

amount of virus taken up by the various organs. Attempts to demonstrate this effect were made with ethyl stearate as the blocking agent. A comparison of the rates of viral clearance in animals treated with 120 mg of ethyl stearate and pyrogen-free saline 24 hours prior to virus inoculation is shown in Figure 6. Little decline in circulating blood titers was noted in the blocked animals 20 minutes after inoculation of virus. At 38 minutes, 90% of the virus was no longer present in the blood, and one hour after injection 98% of the virus had disappeared. Rapid initial clearance of virus from the blood occurred in the control animals; there was 50% disappearance of virus at three minutes, 90% at 10 minutes, and 99.5% at one hour. Percentage viral recovery in organs removed one hour after inoculation from blocked and control animals is shown in Table 16. Greater amounts of the inoculated virus were recovered in each of the organs tested from control animals. Most of the virus was recovered from the liver and lungs of both groups of animals. Percentage uptake in liver, as well as in lungs and spleen of normal animals were 25 and 15 times greater, respectively, than uptake in the corresponding organs of blocked animals. Conversely, circulating blood titers at the time of organ removal were three and one-half times greater in blocked than in control animals. Concentrations of virus in organs from both groups of animals are presented in Table 17. Approximately 60 to 64 times greater concentrations of virus occurred in organs of control animals than in blocked animals.

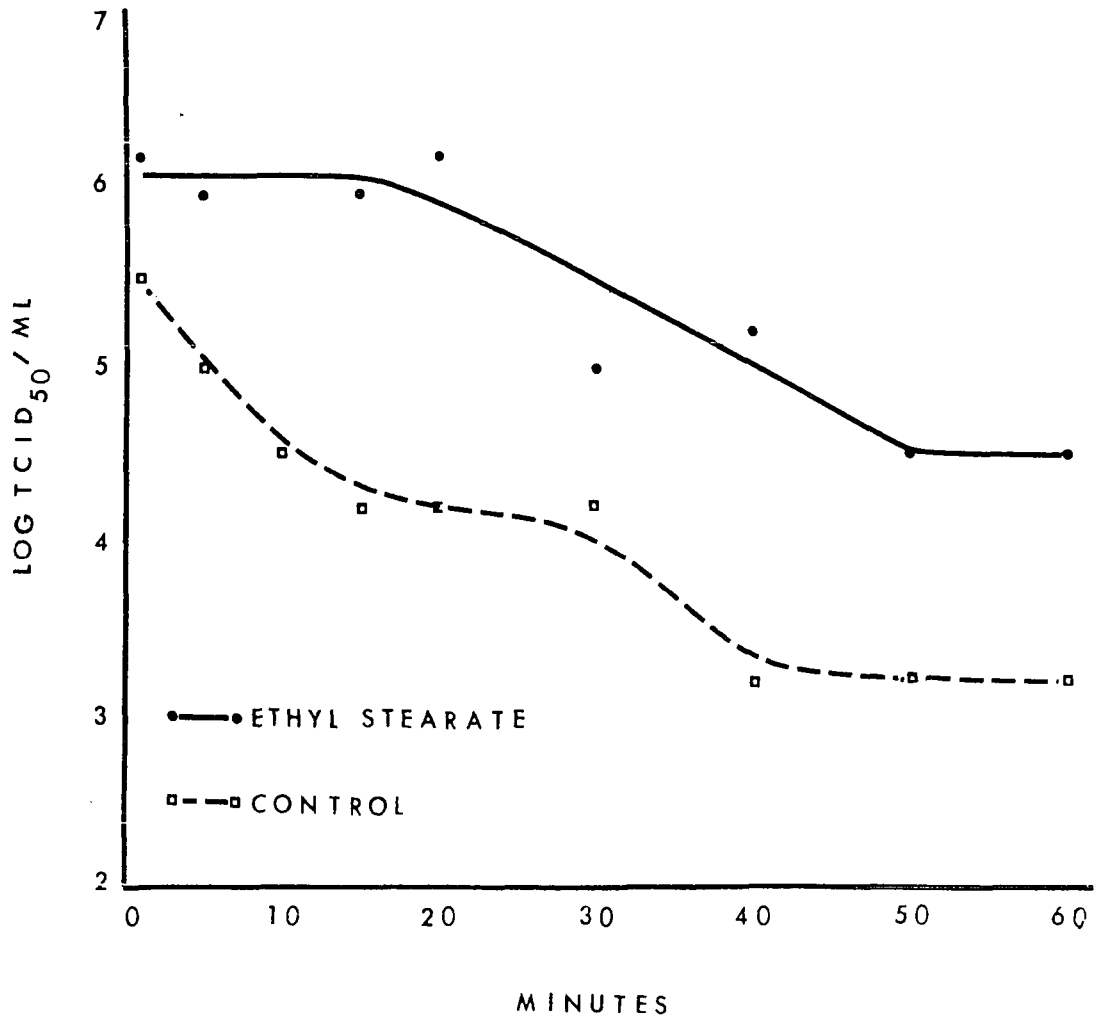

Figure 6. Effect of ethyl stearate on herpesvirus clearance from hamster blood.

TABLE 16

EFFECT OF RES BLOCKADE WITH ETHYL STEARATE ON RECOVERY OF HERPESVIRUS IN ORGANS ONE HOUR AFTER INOCULATION

Organ	Virus Recovered $TCID_{50}$/organ		Percentage Recovered[a]	
	Control	Blocked	Control	Blocked
Liver	270,000	69,000	5.4	0.22
Spleen	15,000	4,800	0.3	0.02
Heart and Lungs	110,000	40,000	2.1	0.13
Adrenals	1,900	250	0.04	0.0007
Kidneys	-	8,000	-	0.03
Blood	14,000	310,000	0.27	0.96

[a]Input virus was $10^{6.7}$ $TCID_{50}$ in the control animals and $10^{7.2}$ $TCID_{50}$ for blocked animals.

TABLE 17

EFFECT OF BLOCKADE WITH ETHYL STEARATE ON UPTAKE OF HERPESVIRUS IN ORGANS ONE HOUR AFTER INOCULATION

Organ	Blood titer		Calculated viral titer in organ due to blood[a]		Actual viral titer in organ		Concentration[b] Factor	
	Control	Blocked	Control	Blocked	Control	Blocked	Control	Blocked
Liver	1600[c]	32,000[c]	1500[c]	25,000[c]	270,000[c]	69,000[c]	180	2.8
Spleen	1600	32,000	120	2,300	15,000	4,800	125	2.1
Heart and lungs	1600	32,000	640	15,000	110,000	40,000	172	2.7
Kidneys	1600	32,000	NR[d]	7,100	NR	8,000	NR	1.1

[a]Blood vol. per g of organ (rat); liver = 0.178; spleen = 0.481; heart and lungs = 0.379; kidneys = 0.278. Lewis et al., 1952.

[b]Actual titers divided by the calculated titer

[c]$TCID_{50}$ per ml.

[d]NR = not run.

The Fate of the Virus in Hamster Organs

Assay of Virus by Titration in Cell Cultures

Tissues were examined at extended time intervals after intravenous inoculation in order to determine whether virus which was localized in organs multiplied and spread to other cells, or was destroyed before replication occurred. Replication of virus within organs was analyzed by infectivity titrations in cell cultures and by fluorescent antibody as well as by conventional histological staining techniques. Groups of adult hamsters were inoculated intracardially with 10^7 $TCID_{50}$ of virus. At each of several time intervals up to 72 hours, at least two animals were bled and organs were removed for preparation of sections or were stored at -70 C for subsequent titration.

Evidence that viral replication occurred in certain hamster organs after intravenous inoculation is presented in Table 18. Viral titers of liver, lungs, and adrenals removed at 24 hours were higher than those obtained at 12 hours. A marked increase was noted in the amount of virus in the adrenals during the 12 hour interval; the titer was 500 times greater at 24 hours than at 12 hours. Smaller increases in viral titers occurred in 24 hours in the lungs and liver (32 and 1.6 times greater, respectively). A decline in viral titers was observed in all organs removed 72 hours after inoculation. This decrease in titer was most noticeable in the liver; no virus could be detected in this organ at 72 hours. Lungs and adrenals removed from the same animals at 72 hours, however, contained virus, although titers were definitely lower than those obtained at 24 hours. No virus was isolated from the blood of any animal at 12 hours, 24 hours, or 72 hours.

TABLE 18

REPLICATION OF VIRUS IN HAMSTER ORGANS FOLLOWING INTRAVENOUS INOCULATION

Organ	Log_{10} $TCID_{50}$ per g of organ		
	12 hr.	24 hr.	72 hr.
Liver	3.5	3.7	0[a]
Spleen	0	0	0
Lungs	2.0	3.5	2.0
Adrenal	2.5	5.2	4.0
Blood	0	0	0

[a]No cytopathic effect in any of the replicate CE monolayers inoculated with a 1:10 dilution of tissue homogenate.

Fluorescent Antibody Stained Sections

Frozen sections prepared from organs removed 30 minutes, 12 hours, 24 hours, and 72 hours after intravenous inoculation of virus were prepared by either the direct or indirect method of fluorescent antibody staining and examined for the presence of viral antigen. Localization of antigen was not observed in sections prepared from organs removed 30 minutes after injection.

Organs removed at 12 hours. Examination of the liver revealed the presence of multiple foci of fluorescing cells throughout the section. Fluorescing cells were not localized around blood vessels, but were scattered without a definite pattern throughout the sinusoidal areas. From two to six foci were present per field (200X magnification), each focus was made up of one to three fluorescing cells. A few fluorescing nuclei were seen, but most of the cells showed only cytoplasmic fluorescence. Two adjacent infected cells are shown in a liver section stained by the direct method in Figure 7. Both the cytoplasm and nucleus of each of these cells exhibited specific greenish fluorescence. A similar section of liver which was treated with specific antiherpes serum before staining is shown in Figure 8. No specific areas of fluorescence were noted. A section of liver removed at 12 hours and stained by the indirect method is presented in Figure 9. In the photograph, at least five foci of fluorescing cells can be counted, and each focus contains one to three cells. The adrenals also showed evidence of viral infection at 12 hours. Groups of fluorescing cells were seen primarily in the cortex of this organ. Foci (four to eight per 200X field) usually consisted of one or two fluorescing cells (Figure 10). Sections of lung and spleen showed

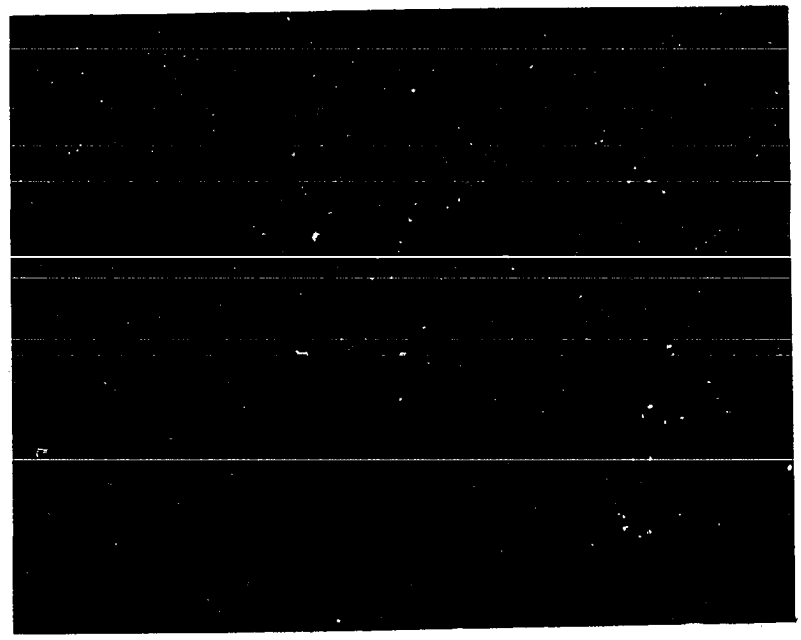

Figure 7. Section of hamster liver removed 12 hours after viral inoculation and stained with fluorescein-labeled globulin by direct method, 400 X. Note apple-green fluorescing cells.

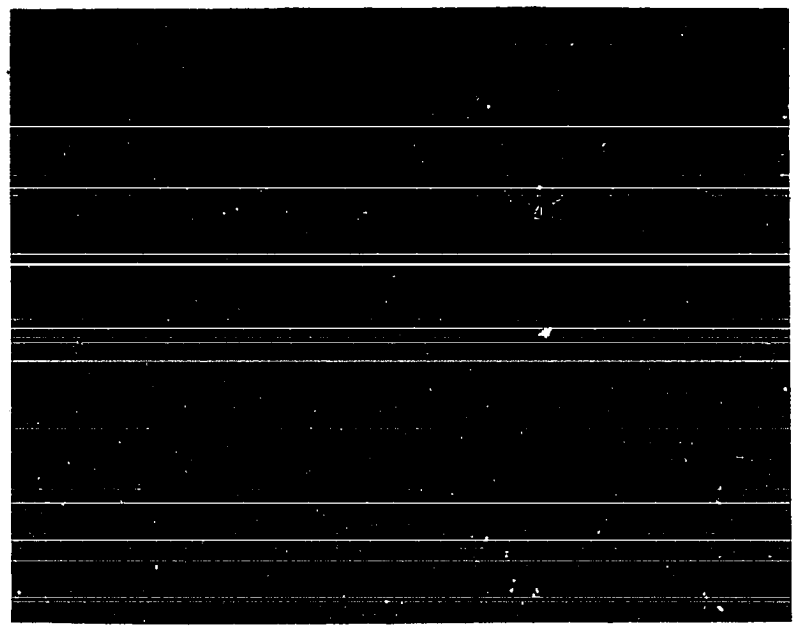

Figure 8. Section of hamster liver removed 12 hours after viral inoculation and treated with specific antiserum before staining with fluorescein-labeled globulin by direct method, 400 X. Note the absence of specific fluorescing cells.

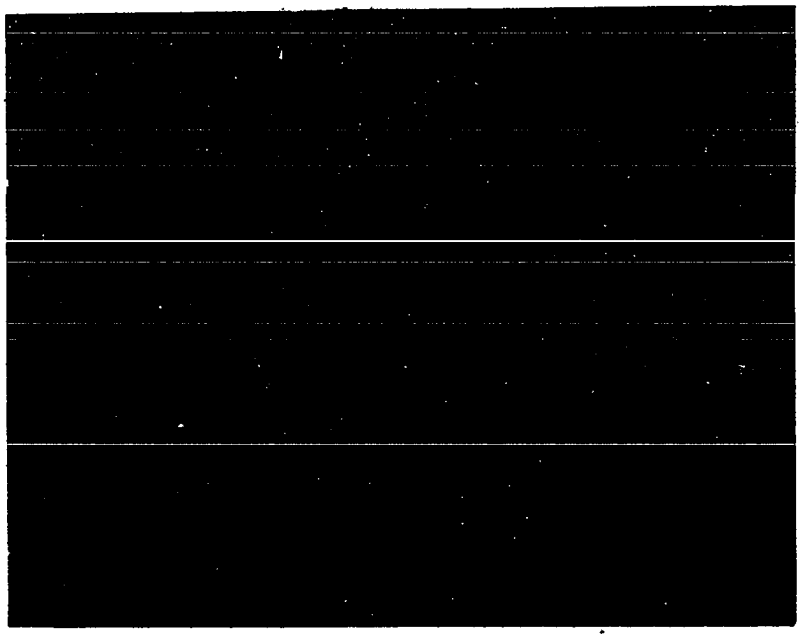

Figure 9. Section of hamster liver removed 12 hours after viral inoculation and stained with fluorescein-labeled globulin by indirect method, 200 X. Note apple-green fluorescing cells.

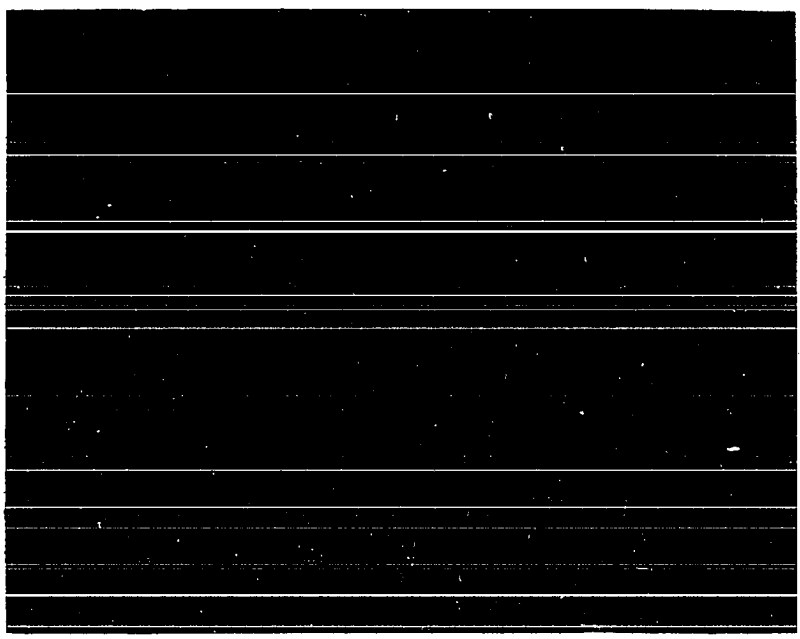

Figure 10. Section of hamster adrenal removed 12 hours after viral inoculation and stained with fluorescein-labeled globulin by indirect method. 200 X. Note apple-green fluorescing cells.

occasional fluorescing cells, but no definite foci were observed.

Organs removed at 24 hours. Foci in the liver had enlarged at 24 hours and many contained 12 to 16 cells (Figure 11). Although foci were larger, i.e., contained more fluorescing cells, they did not appear to increase in number. The few fluorescing cells noted in foci of the cortex of the adrenals at 12 hours had increased markedly at 24 hours. Cells in fluorescing foci had begun to lose their outline and were difficult to count although progression of infection was obvious. Figure 12 shows foci in the adrenal cortex at 200X magnification, while Figure 13 shows a similar area magnified 400X. Fluorescing foci were not observed in the lungs and spleen.

Organs removed at 72 hours. Areas of infection in the liver had decreased in intensity of fluorescence at 72 hours. Cells in the central area of a fluorescing focus appeared to be destroyed and only peripheral fluorescence of low intensity was observed. In the adrenals, fluorescing intensity had faded and areas of destroyed cells were apparent in central areas surrounded by more brightly stained cells at the periphery (Figure 14).

Hematoxylin and Eosin Stained Sections

Examination of hematoxylin and eosin stained sections of organs which were removed 72 hours after viral injection confirmed observations made with immunofluorescent staining techniques. With both procedures areas of necrosis were demonstrated in the adrenal cortex and liver. Typical type A inclusion bodies occurred in cells at the periphery of the necrotic foci (Figure 15). Histologic evidence of infection was not found in the spleen, lungs and brain.

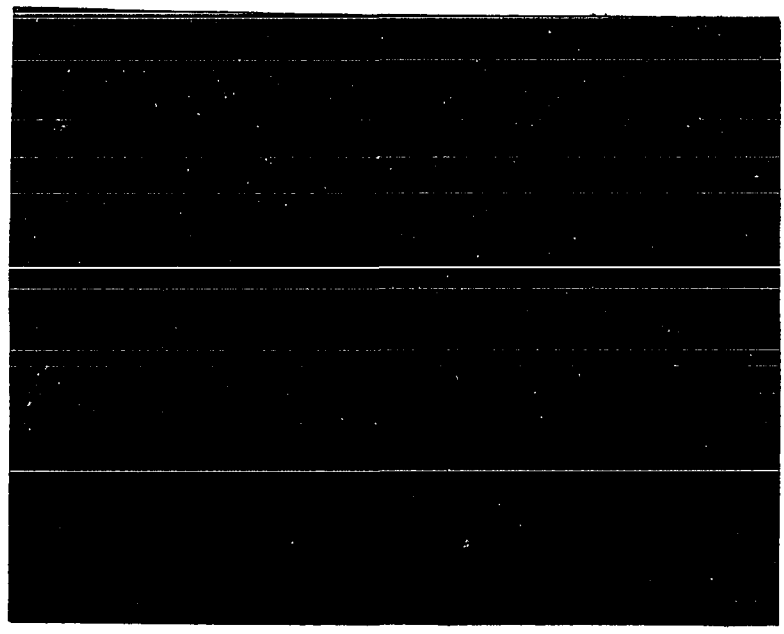

Figure 11. Section of hamster liver removed 24 hours after viral inoculation and stained with fluorescein-labeled globulin by indirect method, 200 X. Note increase in number of fluorescing cells.

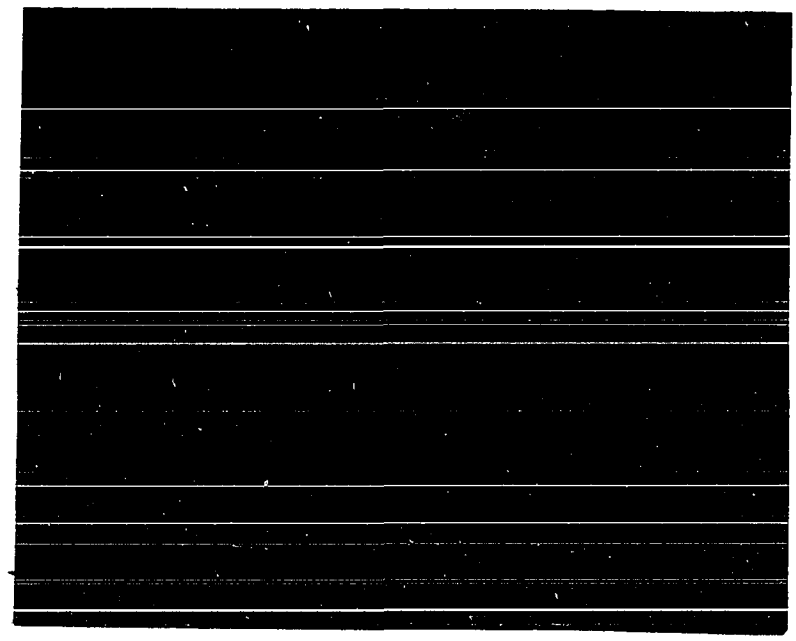

Figure 12. Section of hamster adrenal cortex removed 24 hours after viral inoculation and stained with fluorescein-labeled globulin by indirect method, 200 X. Note large areas of specific fluorescence.

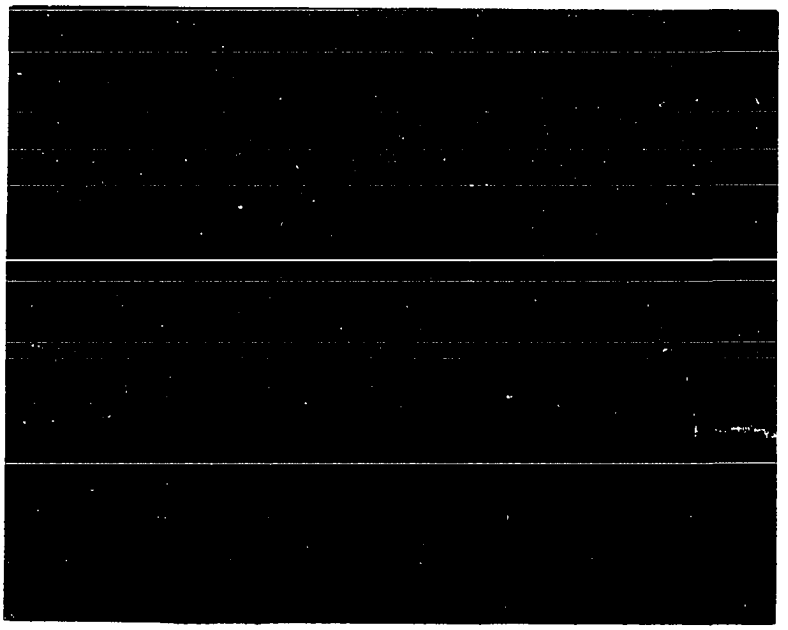

Figure 13. Section of hamster adrenal removed 24 hours after viral inoculation and stained with fluorescein-labeled globulin by indirect method, 400 X.

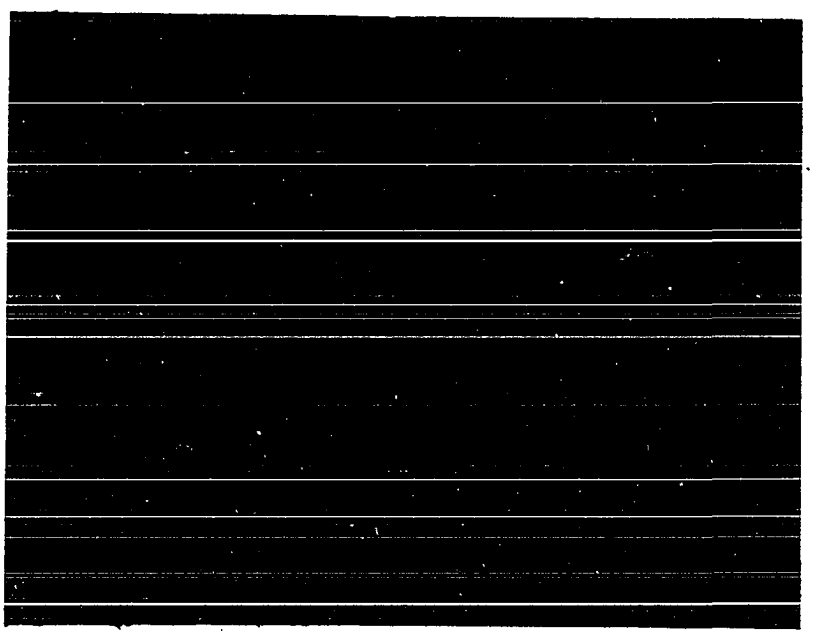

Figure 14. Section of hamster adrenal removed 72 hours after viral inoculation and stained with fluorescein-labeled globulin by indirect method, 400 X. Note areas of necrosis.

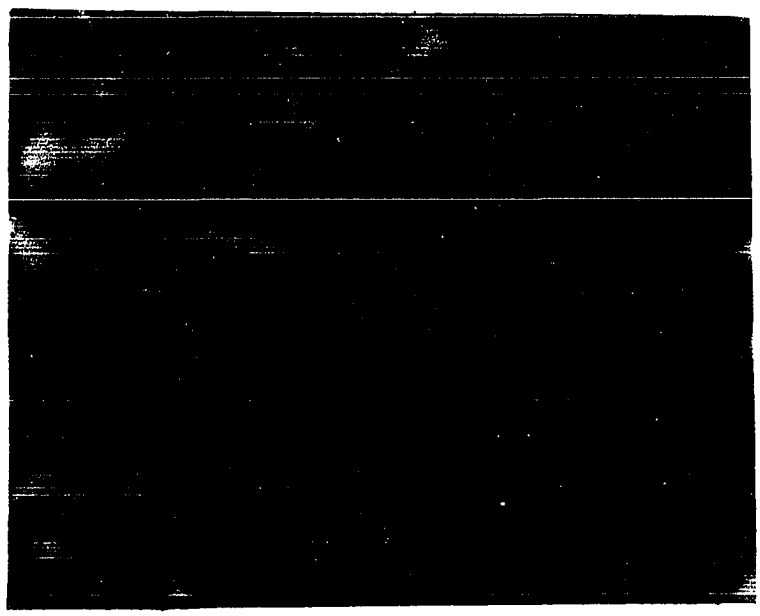

Figure 15. Section of hamster adrenal removed 72 hours after viral inoculation and stained with hematoxylin and eosin, 1000 X. Note type A intranuclear inclusions.

CHAPTER V

DISCUSSION

Determination of virus titers in the circulating blood of hamsters required that samples of blood be withdrawn from the animals at timed intervals following inoculation. It was necessary, therefore, to store the blood samples in a diluent which, in addition to stabilizing the virus, would also prevent clotting. Since heparin had been reported to combine with herpesvirus and prevent its attachment to cells in culture (Nahmias and Kibrick, 1961), the effect of other anticoagulant compounds upon the test strain of virus was investigated. Sodium citrate, in a concentration of 2 mg per ml, did not inhibit herpesvirus and, when added to HLY-10, was satisfactory for sample storage. The chelating agent, EDTA, although known to be an effective anticoagulant, was inhibitory to the test virus under the conditions of these experiments and was not used in these studies.

Titers of antisera produced in rabbits for experimental use were quite variable. It is recognized that the rate and amount of antibody formed in animals as a response to viral infection varies according to the strain of virus used, its state of viability, the route of inoculation, use of adjuvant, and individual as well as species differences. Rabbits have been reported to show considerable individual variation in

immune response to herpesvirus according to Tokumaru and Scott (1964). These workers recommended inducing an actual infection in the animals by corneal scarification followed by live virus booster injections. Using this method they obtained antisera with titers as high as 1:1024 in an occasional rabbit, although titers of 1:128 to 1:256 are the rule following artificial immunization. Corneal infection was not used in the present studies because of the risk of losing the animal by producing encephalitis. The antigen employed was live virus emulsified in Freund's complete adjuvant, since antibody response to many viral antigens have been reported to be greater with an adjuvant (Rhodes and van Rooyen, 1962). Possibly the low antibody titers observed at six weeks following injection reflected slow release of the viral antigen from the site of inoculation due to the effect of the adjuvant. Further booster injections at longer time intervals resulted in significantly higher titered sera (Table 3). Since the crude viral antigen had been prepared in HeLa cells, a heterologous cell line (PK monolayers) was used in neutralization tests. The possibility that anticellular antibodies might inhibit viral cytopathic effects in cell cultures, presumably by blocking the cellular receptors and hence rendering them unavailable for viral attachment, was thus avoided (Quersin-Thiry, 1958).

The activity of the fluorescein-labeled antiherpes globulin preparation was relatively low (Table 4), despite the fact that this conjugate was prepared with the rabbit antiserum which had the highest neutralization of infectivity titer. Other workers have reported testing numerous herpetic antisera before obtaining one which gave adequate intensity of fluorescence. The fluorescing activity of labeled globulin is

apparently unrelated to neutralization of infectivity titers (Johnson, 1964). Brighter staining resulted with the more sensitive, but time consuming, indirect method, an observation also made by other workers who applied this staining technique to viral infected tissue sections (Mims, 1959; Johnson, 1964). Absorption with acetone-dried tissue powders has proved to be an effective means of removing non-specific fluorescence (Coons et al., 1955). In this investigation, absorption of conjugates with both hamster liver powder and rabbit bone marrow powder minimized background fluorescence in tissue sections. Specificity of the labeled conjugates was indicated by: (1) failure of normal tissues to stain, (2) blocking of specific fluorescence in sections pretreated with specific antiherpes serum, and (3) the lack of specific staining in sections treated with labeled normal globulin. In sections stained by the indirect method, substitution of normal unlabeled serum for specific antiserum before treatment with labeled anti-rabbit globulin was an additional control.

The thermolability of herpesvirus and its instability when stored in saline solutions in the absence of protective protein in the suspending medium pose special problems in its preservation and handling. Skimmed milk, egg yolk, and serum-containing diluents have been reported to preserve the activity of herpesvirus for as long as 10 months (Speck et al., 1951). Partially purified and concentrated virus retains activity in distilled water, but not in saline solutions, for short periods of time when stored at 4 C (Munk and Ackerman, 1953). Added protein apparently protects the virus from toxic ions which may be present in saline solutions. Virus suspended in HBSS with 10% inactivated calf serum added as

a protective protein during storage at -70 C, was used in these investigations except for preliminary studies, in which distilled water preparations were used. Virus in distilled water showed little loss in titer when stored for one week at 4 C. Only slight loss of titer of the serum-suspended virus occurred for as long as 75 days at -70 C. By stability tests both preparations proved to be satisfactory for the purposes and conditions of this investigation.

Jenkin and Rowley (1961) reported that both bacteria and carbon particles require nonspecific serum opsonins in order to be phagocytized by the macrophages of the RES. They postulated that depressed clearance of particles and blockade of the RES was due to depletion of serum opsonins by adsorption to large doses of colloidal particles. In this investigation little difference was observed in rates of clearance of herpesvirus which was suspended in 10% inactivated calf or hamster sera, compared to clearance of virus suspended in distilled water. Half of the virus was cleared from the blood stream within five minutes after injection of each of the three preparations, and, in each instance, almost identical levels of residual virus were found to persist throughout the experimental period. Furthermore, no significant difference in organ uptake of virus was noted for the three preparations. Lack of opsonic effect may have been more apparent than real, due to the relatively small mass of viral particles injected. If the postulated serum factors were heat labile their effectiveness would have been destroyed since the serum in which virus was suspended was heat-inactivated. Specific antiserum has been reported to cause a more rapid increase in clearance of some viruses than does normal serum. Brunner et al. (1960) reported that

P^{32}-labeled Newcastle disease virus incubated in antiserum for 30 minutes and inoculated into mice was cleared more rapidly than virus without serum. The effect of specific antiserum is thought to be due to formation of larger aggregates of serum and virus. Larger particles are known to be phagocytized more efficiently than smaller particles (Zilversmit et al., 1952; Benacerraf et al., 1957).

Herpesvirus disappeared rapidly from the circulating blood of adult hamsters for the first 20 minutes after inoculation. At 30 minutes, the clearance rate stabilizes, and there remains in the blood an uncleared fraction of virus, which represents a very small percentage of the amount originally injected. The biphasic nature of the clearance curve appears to be a general feature with viruses. It has been reported to occur with Rift Valley fever (Mims, 1956), ectromelia (Mims, 1959), Newcastle disease and vesicular stomatitis (Brunner et al., 1960), and T_7 bacteriophage and vaccinia (Mims, 1964) viruses. Rates of viral clearance appear to be affected by particle size, as is also true in the case of inert particles (Zilversmit et al., 1952; Dobson, 1957). Large viruses (250 mμ) such as vaccinia and ectromelia have been reported to be removed more rapidly than are smaller viruses, such as the (30 mμ) viruses of poliomyelitis and Rift Valley fever (Mims, 1964). The rapid and efficient clearance of herpesvirus observed in this investigation might have been predicted if one considers its size, which is in the range of 165 to 180 mμ according to measurements obtained by electron microscopy (Epstein, 1962).

Intravenous injection of small amounts of virus resulted in a clearance curve practically identical to that obtained when 99% more

virus was injected. Similar findings have been reported with Newcastle disease virus by Brunner et al. (1960). These workers observed no difference in clearance curves in mice when amounts of P^{32}-labeled virus were increased or decreased by a factor of two. Large doses of both inert particles and bacteria are known to reduce clearance rates, and it has been postulated that the decreased rates are due to saturation of the phagocytic cells of the RES (Benacerraf et al., 1957). Examination of mouse livers by microautoradiographs after the injection of large doses of radio-labeled Newcastle disease virus revealed that many of the phagocytic cells did not contain virus. Brunner and co-workers, 1960, concluded that although large doses of virus were injected, as measured by infectivity titers, the mass of particles injected was relatively small in terms of the capacity of the RES. This interpretation could explain the identical rates of clearance observed in the present studies when doses of virus differing one hundred-fold were inoculated into hamsters.

Persisting fractions of particles in the blood may be due to inhomogeneity in particle size with resulting variable rates of clearance for the different sized particles (Zilversmit et al., 1952; Mims, 1964). Adsorption of virus to cellular elements in the circulating blood may also be a factor which influences clearance (Sheppard et al., 1951; Mims, 1959). Although small amounts of herpesvirus were detected in the cellular fraction of the blood one hour after inoculation, most of the residual virus was present in the plasma. Cell-adsorbed virus alone did not account for the presence of uncleared virus in the blood. Inhomogeneity of particle size may well have been a factor in the persistence of virus in the plasma. Although partially purified virus was used

in these studies, sedimentation and electron microscopic studies reported by Munk and Ackerman (1953) indicated that viral particles in various developmental stages and sizes (naked capsids, one or two envelopes, and aggregates) were present in similar preparations.

Uptake in organs has been described for viral particles as well as for bacteria and inert particles (Mims, 1959; Brunner et al., 1960; Byatt and Rasmussen, 1964). The percentage of virus recovered in the liver and spleen 90 minutes after inoculation was less than 4% of the amount inoculated. Computations of the degree of concentration of virus within these organs indicated that virus had been taken up in noticeably higher amounts than was present in the blood. It was possible that the relatively small amount of virus detectable in organs at 90 minutes could be explained by adsorption of virus to cells and entry into the eclipse phase. At this stage in the replication cycle the virus cannot be detected by infectivity titrations. Studies of the growth of herpesvirus in embryonated eggs and in monolayers of rabbit corneal cells (Scott et al., 1953a; 1953b) showed that initial adsorption of virus to cells is rapid; 99% is adsorbed in one hour. The adsorbed virus quickly enters the latent stage which lasts from two to 12 hours. The length of this stage is dependent upon the host infected and the number of infectious particles in the inoculum. Significantly higher recoveries of virus were obtained from the lungs, liver, and spleen, at 10 minutes than at 90 minutes. In contrast, the degree of concentration of virus within these organs at 10 minutes was lower than that occurring at 90 minutes. This possibly was due to the relatively higher circulating blood titer at 10 minutes than at 90 minutes. Evidence that early adsorption and

entry into the eclipse phase had occurred was obtained by titrating organs at periods from five through 90 minutes (Figure 4). Approximately 75% of the input virus was recovered at five minutes, and 58% was in the liver. Titrable virus in organs decreased progressively during the test period. This decrease was interpreted to mean that virus had been taken up in organs; and after uptake, it was either in the eclipse phase, or was destroyed within the organs. The progressive increase of virus within organs indicated that at least some of the adsorbed virus was not destroyed.

Inactivation of 95 to 98% of herpesvirus by normal hamster serum confirmed the observations that Finkelstein et al. (1959) made with rat serum. Reversal of viral inactivation, by heating the serum or by adsorption with zymosan at 16 C, indicated that the inhibitor was properdin. Inactivation was not observed to occur when virus was incubated with undiluted virus. Conversely, when serially diluted virus was added to normal serum and incubated, marked and progressive viral inhibition occurred as time of incubation was increased. Similar findings were reported by Finkelstein et al., 1959. Definite, but reduced, inhibition was observed by these workers in serum mixed with undiluted virus. Rat serum, which is known to contain high concentrations of properdin (Pillemer et al., 1954), was used in the cited experiments. Although titers of properdin in hamster sera were not determined, lower concentrations of properdin in these sera possibly could explain the lack of inhibition observed when undiluted virus was tested. Increased inhibition of diluted virus by normal serum was postulated by Finkelstein et al. (1959) to be due to the presence of host substances in undiluted viral pools which interacted with the serum inhibitor and prevented its action. Dilution of the

virus, and concurrently the contaminating host material, would act to minimize or remove the interfering material and leave the virus susceptible to the effect of the serum inhibitor. The effect of properdin upon viruses *in vivo* is not known. Sulkin *et al.* (1957) treated rabbits possessing known properdin titers with zymosan, and later injected bacteriophage intravenously. Amounts of phage isolated in animals pretreated with zymosan were three to tenfold higher than in untreated animals. These workers reported that zymosan "reduced the efficiency of mechanisms responsible for phage clearance." However, they did not determine if higher levels of phage in zymosan treated animals were due to neutralization of the properdin system, or were due to blockade of the phagocytic function of the RES. The effect of properdin upon disappearance of the virus from the blood of animals in the present studies is not known. That plasma inhibitors did not destroy all of the virus inoculated is apparent, since infection and spread in organs was shown to occur. In addition, indirect evidence that properdin inactivation did not occur *in vivo* may be inferred from clearance studies in blocked animals. Levels of infective virus did not decrease significantly for at least 20 minutes in animals pretreated with ethyl stearate and inoculated with virus 24 hours later, a time when all of the lipid is presumably removed from the blood. Rate studies on serum inhibition *in vitro* (Figure 5) with diluted virus, showed that 69 to 92% of the virus had been inactivated at 20 minutes depending on the degree of viral dilution. No loss of this magnitude was found to occur in blocked animals in 20 minutes when virus was diluted approximately one to ten in the circulating blood plasma.

Ethyl stearate is reported to depress clearance by affecting

only fixed macrophages of the liver and spleen without apparent changes in other cellular systems or organs (Cooper and Stuart, 1962). Blockade of the phagocytic cells of the RES was indicated in the present study by decreased rates of clearance and by lower recoveries of virus in organs. Virus was concentrated in organs of control animals approximately 60 times that in blocked animals. Less virus was recovered and concentrated in organs of blocked animals despite the presence of higher levels of virus in the circulating blood at the time the organs were removed. Levels of residual virus remained high for at least 20 minutes after inoculation. A marked decrease occurred later, and one hour after viral injection, 98% of the virus could not be detected. Levels of virus in the blocked animals were always higher than those which occurred in controls. Lower blood titers noted for the treated animals after 20 minutes suggested that only a partial block of the RES was accomplished, or that virus which disappeared was not phagocytized by RES macrophages, but was adsorbed to vascular endothelium or to other tissues. In support of the latter hypothesis, Benacerraf et al., 1959, reported that carbon clearance was reduced after treatment of mice with Thorotrast. Non-phagocytized particles remained in the blood in high concentration and carbon was found to be localized in the capillary endothelium of these animals. Even if phagocytosis or adsorption to tissues did not occur, virus present in the circulating blood for extended periods of time would be expected to disappear as the result of thermal inactivation. The half-life of herpesvirus is reported to be one and one-half to three hours at 37 C (Powell, 1959; Farnham and Newton, 1959), and about three hours at 31 C (Scott et al., 1961). The finding that blockade of

the RES resulted in decreased uptake of virus in organs as compared to that observed in untreated animals, provided further evidence that phagocytosis of herpesvirus by the macrophages of the RES had occurred. This observation parallels that of Brunner et al., 1960, with Newcastle disease virus in mice. These workers blocked the phagocytic function of the RES with Thorotrast and noted a marked decrease in rates of viral clearance and in uptake of virus in organs.

Mims (1964) assigned an important role to the macrophage system of cells in virus infections. Since macrophages monitor the various body compartments of animals, they are in a position to control the entry of viruses into organs. The susceptibility of animals to viruses may be determined at the macrophage level. Virus which is taken up in macrophages may be destroyed, or it may infect the phagocytizing cells with subsequent transfer to surrounding cells. In addition, the macrophage may take up the viral particle and transfer it to other cells without an initial replicatory stage within the phagocyte. Virus, which was cleared from the blood and concentrated in organs in this study, was presumed to be taken up by macrophages, particularly those in the liver, spleen, and adrenals. Direct evidence of the presence of viral antigen in macrophages was not obtained in fluorescent antibody studies of sections of organs removed at 10 and 30 minutes after injection of large doses of virus. Replication of virus in the liver, adrenals, and lungs was indicated by increased viral titers in the organs removed 24 hours after inoculation compared to those obtained at 12 hours. Viral multiplication was particularly noticeable in the adrenals, and the peculiar susceptibility of this organ to infection with herpesvirus has been noted by Hass (1935), who first

described its occurrence in newborn human beings. Herpetic necrosis in the adrenals of rabbits and pregnant hamsters also was noted by Smith (1931) and by Ferm and Low (1965), respectively. In the last study, histologic evidence of extensive destruction of the adrenal cortex was present in all the injected adult animals. Small areas of necrosis were also found in the liver. Despite the severe involvement of the adrenal glands and lesser degrees of infection in other tissues, the animals survived and delivered normal offspring. Replication of virus was not noted in infectivity titrations of spleen in the present study, although uptake of virus in this organ was shown to occur in clearance studies; but to a lesser degree than in the liver. The susceptibility of splenic macrophages to the virus may be different from those in other organs. An unexpected finding was viral replication in the lungs following intravenous inoculation, since this organ does not contain macrophages which monitor the circulating blood as do organs of the RES. Clearance studies also showed that virus was taken up and concentrated within this organ. Detectable virus at 12 and 24 hours could not have been due to the large volume of blood in this organ, since virus was not present in the circulating blood at this time. Titers of virus in all organs were reduced at 72 hours as compared with those observed at 24 hours. This was particularly noticeable in the liver since no infectious virus was obtained at 72 hours in this organ. Adult hamsters inoculated with herpesvirus by the intravenous route usually do not die. Symptoms of infection may or may not occur after inoculation. Animals with symptoms usually recover after a few days and continue to gain weight and appear normal. Decline in titers of infected organs was, therefore, not surprising.

Confirmatory evidence of viral replication in the liver and adrenals at 24 hours post-inoculation was obtained in fluorescent antibody studies of sections of these organs. Infected foci in the liver and adrenals became progressively larger in time as shown by specific fluorescence in increased numbers of parenchymal cells. Spread of virus from infected cell to adjacent unifected cell appeared to increase centrifugally. At 72 hours, the decreased numbers of fluorescing cells and diminished intensity of fluorescence plus lower infectivity titers in the organs indicated that multiplication and spread of the virus was reduced. Virus was not isolated from the blood of the animals at any time during the extended studies in organs. This can be explained by the nature of the limited infection in adult animals, and the known ability of this virus to infect neighboring cells through direct cell to cell viral transmission (Hoggan and Roizman, 1959).

Johnson (1964b) has recently reported that resistance of mice to infection with herpesvirus develops with age and occurs at the level of the macrophage system. Macrophages from both adult and suckling mice were easily infected <u>in vivo</u> and <u>in vitro</u>, and no difference in development of the viral antigen was apparent during the first growth cycle. Macrophages from suckling mice were capable of transmitting infective virus to other cells in contact with them, whereas macrophages from adult animals could not. The occurrence of a macrophage "barrier", as described by Johnson in adult mice, was not observed in the adult hamsters used in this study. The progressive spread of viral antigen to adjacent cells during the course of the infection, in the absence of virus in the circulating blood, would appear to rule out limitation of viral spread at

the macrophage level. An alternate explanation for the limited spread of viral infection in adult animals is suggested in recent reports by Baron et al. (1966a, b). These workers showed that production of interferon is a rather general response by animals to viremias. Circulating interferon was shown to be produced within a few hours after onset of viremia and protected various organs from viral infection. Evidence that production of interferon is related to the age of the animal was presented by Heineberg et al. (1964). Lethal infections resulted when neonatal mice were inoculated with Coxsackie B virus. On the other hand, adult animals were found to be resistant to infection with this agent, and resistance was reported to be due to reduced viral replication and enhanced interferon production in infected tissues.

CHAPTER VI

SUMMARY

Following intravenous injection of large doses of herpesvirus into adult hamsters, sequential titrations of residual infective virus in the circulating blood revealed that 90% had disappeared within a period of 10 minutes. Half-times of viral disappearance occurred within five minutes after inoculation, and by 30 minutes, 99% of the virus had disappeared. Low levels of virus persisted in the blood for at least 90 minutes. Identical rates of clearance were observed when 99% less virus was injected except that the titer of the uncleared viral fraction with the smaller inoculum was lower. No difference in rates of clearance was noted for virus suspended in distilled water, and in homologous or heterologous serum.

Seventy-three percent of the virus which persisted in the circulating blood at 60 minutes was recovered in the plasma fraction. Ten percent remained with a fraction containing red and white blood cells after five washings. Viral activity was not observed in a fraction composed of washed platelets.

Titrations of organs five minutes after viral inoculation revealed that most of the infectious virus was present in the liver, spleen, and lungs. Titratable virus in organs removed at various time intervals after

inoculation decreased progressively, although the amount of virus in the organs was greater than that in the blood.

The disappearance of virus from the bloodstream proved to be due to uptake by macrophages of the reticuloendothelial system as demonstrated by blocking the function of the phagocytic cells with ethyl stearate. As a result of such blockade, there was decreased clearance and less concentration of virus within organs.

In vitro inhibition of infectivity of 95 to 98% of herpesvirus was found to occur when diluted virus was incubated for 90 minutes with pooled normal hamster serum. The rate of inhibition icreased from 92% at 10 minutes to 98% at 90 minutes when serum was mixed with serially diluted virus. When the virus was diluted 1:10 with serum before incubation, maximum inhibition (70%) occurred at 10 minutes and did not increase throughout the test period. Inhibition was not observed when undiluted virus was used. Lack of inhibition in either zymosan treated or heat-inactivated serum indicated that the inhibitor was properdin.

When compared to those obtained at 12 hours viral titers of organs removed 24 hours after viral inoculation were 500 times greater in the adrenals, and 32 and 1.6 times in the lungs and liver, respectively. A decline in recoverable virus occurred in all organs at 72 hours. Virus was not detected in the spleen and blood at 12 hours nor at any time thereafter.

Examination of tissues by immunofluorescent staining showed that numbers of fluorescing cells per infected focus increased progressively from 12 to 24 hours, although numbers of foci did not increase. At 72 hours, increased numbers of fluorescing cells were not observed, intensity

of fluorescence was decreased, and evidence of cellular necrosis at centers of foci was present. Fluorescing foci of infected cells were not seen in the lungs or in the spleen.

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