

VACCINIA VIRUS IN ACTIVATED MACROPHAGES

ABORTIVE REPLICATION OF VACCINIA VIRUS
IN ACTIVATED RABBIT MACROPHAGES

By

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ABSTRACT

Macrophages obtained from animals infected with intracellular parasites are activated with respect to their ability to inhibit the replication of the parasite. While normal rabbit macrophages support the replication of vaccinia virus at levels of 2 to 3 logs, macrophages obtained from the peritoneal cavity of rabbits infected 9 to 12 days earlier with vaccinia virus are activated and will not support virus replication. The fate of vaccinia virus in activated rabbit macrophages was studied in order to characterize the abortive infection of the virus within the activated macrophage. Vaccinia virus adsorption was measured using radioactively labelled virus and was similar with both normal and activated macrophages but was lower than the amount of virus which adsorbed to Vero cells. Maximum adsorption took place during the first 10 minutes of incubation. A significant amount of the vaccinia virus which had adsorbed to the cells eluted from the cells during further incubation. However, the virus elution curves for activated and normal macrophages were similar. Virus uncoating was measured by infecting with ^3H thymidine labelled vaccinia virus and then detecting DNase sensitive, TCA soluble counts. Vaccinia virus was able to uncoat to a similar degree in both activated and normal macrophages. Maximum virus uncoating took place one to four hours after adsorption and was approximately 55% of the virus which was adsorbed to the cells. DNA synthesis in vaccinia virus infected cells was detected by pulse labelling with ^3H thymidine. A burst of DNA synthesis at 3 to 6 hours after infection took place in both activated and normal macrophages infected with vaccinia virus as well as infected Vero cells. The pattern of vaccinia virus antigen production in activated and normal macro-

phages was identical as detected by immunofluorescence and immunodiffusion. Autoradiographs of SDS-PAGE gels of lysates of infected cells pulsed with ^3H amino acids demonstrated that most of the polypeptides formed within the infected macrophages were identical. However, at least three polypeptides present in the activated macrophages infected with vaccinia virus were absent in the infected normal macrophages and at least one polypeptide present in the virus infected normal macrophages was absent in the virus infected activated macrophages. Pulse chase experiments failed to demonstrate that the differences in polypeptide synthesis in activated and normal macrophages infected with vaccinia virus were due to differences in posttranslational cleavage. Lack of virus particle production in activated rabbit macrophages infected with vaccinia virus was the major detectable defect in the viral replicative cycle. Virus particles were detected by centrifuging on a continuous sucrose gradient cell lysates of virus infected cells labelled with radioactive thymidine or amino acids. No virus particles with the size and density of vaccinia virions were detected in lysates of activated macrophages infected with vaccinia virus. Virus particles were present in normal macrophages and Vero cells after vaccinia virus infection.

It appears that the inhibition of production of infectious virus in activated macrophages is mediated by mechanisms other than those induced by interferon. Vaccinia virus DNA and protein were synthesized in activated macrophages. This is in contrast to numerous previous studies which have shown that no vaccinia viral DNA or protein is synthesized in interferon treated cells. Pretreatment of normal rabbit macrophages with tissue culture interferon (type I) did not block the replication of vaccinia virus. Pretreatment of normal macrophages with serum from a poly (I)-poly (C) injected rabbit reduced the replication of vaccinia virus but the characteristics of the abortive infection appeared different than in activated macrophages. Therefore, it appears that interferon is not involved in the inhibition of viral replication by activated macrophages.

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INTRODUCTION

Macrophages play a major role in protecting the body against infectious agents. Several diverse functions may be exhibited in response to the infecting agents. One of the most vital functions performed by macrophages is the phagocytosis of foreign material. Macrophages also function as one of the principal cells in the inflammatory process. The macrophage is necessary in the formation of the specific immune response to many antigens. Recent evidence has also demonstrated that activated macrophages may act alone to destroy some bacterial, protozoal, and viral intracellular parasites as well as lyse or inhibit the growth of tumor cells.

1. Characteristics of Macrophages

The macrophage is the mature member of the mononuclear phagocytic cell line (Cohn, 1968). The macrophage precursor cells develop in the bone marrow as promonocytes. There they give rise to monocytes which circulate briefly. The monocytes randomly leave the blood stream or attach to the wall of the sinusoids where they undergo a transformation into inflammatory or tissue macrophages (Yang and Skinsnes, 1973; Cohn, 1975). These tissue macrophages may reside for a relatively long life span in organs such as the liver where they are called Kupffer cells, in the lung where they are known as alveolar macrophages, and in the spleen and lymph nodes where they are termed sinusoidal or dendritic macrophages. In the central nervous system, the tissue macrophages are known as glial cells, in connective tissue they are called histiocytes, in bone they are referred to as osteoclasts, and in the peritoneal cavity they are known as peritoneal macrophages (Cohn, 1968).

The maturation from monocyte to macrophage involves both structural and functional alterations (Cohn, 1975). During the process, the cell visibly increases in size and takes on a more complex looking cytoplasm (Ebert and Florey, 1939). The cytoplasmic granules (presumably

lysosomes) increase in number and the levels of dehydrogenases and hydrolytic enzymes rise to above normal. The cells also become more phagocytic (Cohn, 1968).

Macrophages are not fully mature in newborn mice (Yang and Skinsnes, 1973), human (Horsmanheimo and Virolainen, 1974; Sullivan, 1975) and rat (Fieldsted and McIntosh, 1971). The development of mature macrophages appears to correlate with the appearance of cell mediated immune responsiveness (Yang and Skinsnes, 1973). Experiments have shown that neonatal thymectomy of mice will inhibit the formation of mature macrophages. This was demonstrated by a suppressed response of the peritoneal macrophages of neonatally thymectomized mice to intraperitoneal infection with Mycobacterium lepraemurium and to granuloma formation (Yang and Skinsnes, 1973).

Macrophages are somewhat heterogenous in appearance. The cells usually contain an indented nucleus amid a moderate amount of cytoplasm. The cells are large compared to other lymphoid cells and contain numerous dense cytoplasmic granules. Most of these dense granules are considered to be secondary lysosomes which contain phagocytized material and endogenously synthesized hydrolytic enzymes (Cohn, 1968). The hydrolytic enzymes are synthesized in the endoplasmic reticulum and then transferred to the Golgi apparatus where they are packaged into small Golgi vesicles also known as primary lysosomes. The Golgi vesicles then fuse with a phagosome and release the hydrolytic enzymes into the newly formed secondary lysosome (Cohn and Hirsch, 1966). In most cases, both pinocytic and phagocytic vacuoles readily fuse with both primary and secondary lysosomes (Cohn, 1975). However, it has been observed that phagocytic vacuoles containing virulent Mycobacterium tuberculosis (Armstrong and D'arcy Hart, 1971) or Toxoplasma gondii (Jones and Hirsch, 1972) do not fuse with the lysosomes of cultured mouse peritoneal macrophages. This defect in membrane fusion may be responsible for the survival of these virulent intracellular parasites within the macrophage.

The level of lysosomal enzymes within the macrophage appears to

be related to the activity of the macrophage. Increases in the levels of lysosomal enzymes have been shown in mouse peritoneal macrophages after the endocytosis of degradable substances (Pesanti and Axline, 1975). Since colchicine (10^{-6} M) inhibited the lysosomal enzyme induction by both phagocytic and pinocytic stimuli, intact microtubules are thought to be involved in the enzyme inductions. Colchicine, however, had no effect on the digestion of phagocytized bacteria. This suggests that microtubules are not required for fusion of phagosomes and lysosomes (Pesanti and Axline, 1975).

The ability of macrophages to phagocytize particulate material and to adhere to glass or plastic surfaces are two of the most important characteristics which distinguish the macrophage from other mononuclear lymphoid cells. Phagocytosis has been divided into two phases: attachment and ingestion (Rabinovitch, 1967). The firm attachment of a particle to the cell surface does not necessarily lead to its interiorization. Attachment to the macrophage increases linearly with the concentration of particles present, does not require serum or divalent cations and is less sensitive to temperature than the ingestion phase. Ingestion requires the presence of serum and divalent cations and is temperature dependent (Rabinovitch, 1967). The interiorization of antibody coated sheep erythrocytes by mouse peritoneal macrophages has been studied by Tizard and Holmes (1974) using a scanning electron microscope. Initial attachment was observed as an area of adhesion between the macrophage and the erythrocyte. The area of attachment increased as a thin membranous process appeared to flow over the erythrocyte surface until the entire red cell surface was covered. After complete enclosure, the erythrocyte appeared to move toward the center of the macrophage until there remained only a raised area on the macrophage surface. Other studies have shown that when macrophages ingested large numbers of particles, they interiorized up to 60% of their own plasma membrane in the process (Cohn, 1975). After such intense phagocytic activity, these cells rounded up and ceased further phagocytic or pinocytic activity for the next 5 to 6 hours. During this time, they began to synthesize

enough new plasma membrane to replace the amount they had interiorized. This new membrane synthesis required protein and RNA synthesis as well as an extracellular source of cholesterol (Werb and Cohn, 1972).

Phagocytosis by macrophages is an active process and depends on energy supplied by glycolysis (Cohn, 1968). This was shown by the blockage of phagocytosis with glycolytic inhibitors such as fluoride and iodoacetate. Particle uptake has also been shown to increase the rate of these energy producing pathways (Gudewicz and Filkins, 1974).

The coating of particles with specific antibody makes them more susceptible to phagocytosis. In vivo studies have suggested that IgM may be a more effective opsonin than IgG but the role of complement mediated alteration of the particle in this system can not be determined under in vivo conditions. Under in vitro complement free conditions, IgG appears to be a more efficient opsonin (North, 1970). The role complement plays in phagocytosis is still not clear. The components of complement may act like an opsonin or may be useful only in stabilizing the antigen-antibody complex (Humphry and White, 1970).

The microfilaments and microtubules of macrophages appear to play a necessary role in phagocytosis. Both colchicine (10 μM) and cytochalasin D (.5 μg/ml) blocked phagocytosis by mouse peritoneal macrophages (Mimura and Asano, 1976). When the two drugs were combined together, they had a synergetic effect on the inhibition of phagocytosis.

The attachment and spreading of macrophages on a glass surface has been suggested to be an attempt by the macrophage at phagocytosis (North, 1970). After settling on a glass surface, the macrophage sends out fine filamentous processes around itself. Plasma membrane then fills in the spaces between the processes to form a thin cytoplasmic film around the central macrophage (Tizard and Holmes, 1974). Macrophage spreading is favored by slightly acidic pH, trypsin and pronase, dithiothreitol and divalent cations (Cohn, 1975).

The surface of macrophages as well as monocytes contain receptors for IgG and C'3 (Huber et al, 1968). These receptors are useful in

the binding of immune complexes to the surface of the macrophage (Arend and Mannik, 1973). The number of IgG receptors was increased by repeated stimulation with complete Freund's adjuvant (Arend and Mannik, 1973). The increased number of IgG binding sites led to increased binding of soluble immune complexes. The macrophage receptors for IgG can be redistributed into polar caps and then removed by divalent anti-Ig antibody (Loor and Roelants, 1974). Complete removal of all the surface immunoglobulin did not occur. Surface bound antigen such as tobacco mosaic virus or horse spleen ferritin could also be bound and then endocytosed. However, after four days of culture, a portion of the antigen still remained at the cell surface. It was not known if the presence of surface antigen was due to escape from polar capping, surface reexposure of endocytosed material or exocytosis and then reattachment of the antigen. It appears then that the fluid mosaic model of membrane structure applies to what is known about the macrophage plasma membrane.

The macrophage secretes many extracellular factors into its environment. These include interferon, transferrin, complement, and collagenase (Alexander, 1976). The macrophage also produces and secretes a large amount of lysozyme. Under in vitro conditions, 85 to 90% of the total amount of macrophage synthesized lysozyme is extracellular (Cohn, 1975). Activated macrophages produce and secrete plasminogen activator. The secreted extracellular factors may be important in destruction of extracellular microorganisms and tumor cells, as well as in modifying the environment of tissue macrophages and in influencing the behavior of neighboring cells.

While lymphocytes have been shown to be extremely sensitive to x-irradiation, most macrophage functions are relatively x-ray resistant (Geiger and Gallily, 1974a). Unstimulated macrophages from lethally or sublethally x-irradiated mice had normal rates of phagocytosis and degradation of a particulate antigen (Shigella). The irradiated macrophages also had normal or elevated levels of DNA and RNA synthesis. The uptake of choline was enhanced by x-irradiation as were the levels of acid phosphatase and cathepsin D. However, the irradiated macrophages

were not able to induce an immune response to Shigella. This was demonstrated by incubating macrophages from normal or x-irradiated mice with Shigella for one hour. The macrophages were washed and injected into another irradiated mouse along with normal lymph node lymphocytes. The anti-Shigella response was then tested 7 days later and proved to be absent (Geiger and Gallily, 1974a). Electron microscopic observations of in vivo irradiated peritoneal macrophages have shown that they are incapable of forming close physical interactions with normal lymphocytes (Gallily and Ben-Ishay, 1974). This failure of irradiated macrophages to interact closely with lymphocytes may be responsible for their inability to induce antibody production. Scanning electron microscopy has also shown that x-irradiated macrophages are morphologically altered. X-irradiated macrophages contained numerous small holes in their outer membrane (Geiger and Gallily, 1974b). It is not known if these holes represented surface damage or a type of normal surface opening which had not closed.

When macrophages are cultured in vitro for an extended period of time, they often elongate, produce long processes and take on a fibroblast-like appearance (Hirt and Bonventre, 1973). There has been a controversy as to whether the macrophages have been transformed into the fibroblast-like cells or whether the appearance of the cells is due to the overgrowth of a few contaminating fibroblasts. Most of the detailed studies of macrophages in culture have supported the theory that macrophages change into fibroblast-like cells under certain in vitro conditions. Early studies, in 1926, by Carrel and Ebeling (1926) of cultured chicken blood monocytes grown in plasma and embryo juice, showed that after one day in culture, the monocytes had increased in size and taken on a macrophage-like appearance. By four days of culture, these same cells had become long and slender and had lost the undulating membrane along the sides of the cell. Schwartz (1967) has reported that after one day of culture in a rich medium containing 50% ascites fluid and 15% calf serum, peritoneal macrophages from normal guinea pigs took on an elongated fibroblast-like appearance. Cytophilic antibody did not

attach to the cells after they had elongated. Mouse peritoneal macrophages have been cultured for over 200 days in special enriched media (Chang, 1964; Smith and Gokcen, 1971). Many of the cells produced long fibroblast-like processes. Hirt and Bonventre (1973) have presented photomicrographic evidence that the fibroblast-like cells in macrophage cultures arise from dedifferentiated macrophages. Peritoneal macrophages from normal guinea pigs were cultured for 4 days with a suspension of zymosan particles. Zymosan is resistant to digestion by macrophage hydrolytic enzymes. The zymosan was then removed and the cells were further cultured in medium containing 20% fresh guinea pig serum. To test the phagocytic ability of the cultured cells, Staphylococcus aureus was added for two hours and then the cells were stained. At 7 days of culture, there was a mixture of phagocytic macrophages and non-phagocytic fibroblasts. The fibroblast-like cells contained zymosan particles which indicated that they had once been phagocytic but could no longer perform phagocytosis. The number of fibroblast-like cells increased with the age of the culture until they became the predominant cell type.

II. Role of Macrophage in Inflammation

Macrophages and polymorphonuclear leucocytes (PMN) are the major phagocytic cells at inflammatory sites (Dannenberg, 1975). These two cell types migrate simultaneously from the blood stream to the site of inflammation. The PMN's are numerous during the first 12 hours but then die off leaving the macrophages as the predominant cell type during the remainder of the inflammation. Monocytes leave the circulation and migrate to the site of inflammation where, under the proper stimulus, they are transformed into active macrophages. Chemotactic agents released at the site of inflammation appear responsible for much of the cellular migration. Among these agents are the chemotactic factors C3a and C5a released during the activation of complement, partly denatured protein from dead cells at the site of inflammation, and lymphokines such as macrophage chemotactic factor and migration inhibition factor (Dannenberg, 1975). During delayed hypersensitivity reactions, the lympho-

cytes, through the action of their lymphokines, are responsible for the immunological specificity of the inflammatory response.

During short lived acute inflammations, there is a rapid cell turnover with a swift destruction of the irritant. During chronic inflammation, the irritant persists along with a large population of macrophages. Spector and Ryan (1970) have demonstrated that the inflammatory macrophage population may be maintained by three different mechanisms. The population depended on mitotic division of existing macrophages, macrophage longevity, and continued migration from the circulation.

Dead phagocytes accumulating at the site of inflammation may act not only as chemotactic agents but also as sources of glycolytic energy (Gudewicz and Filkins, 1974). It has been demonstrated that glycogen rich leucocyte cytoplasmic debris in inflammatory fluid was a potential source of fuel for maturing macrophages.

The macrophage's main function during inflammation is the engulfment and sequestration or digestion of foreign or altered-self substances. After phagocytosis and fusion of the phagosome with the lysosomes, little is known about the fate of infectious agents (Dannenberg, 1975). Some types of microorganisms may be killed directly by the macrophage's lysosomal enzymes. Lipases may remove the outer cell wall of Gram negative bacteria and lysozymes may then dissolve the remaining mucopolysaccharide layer. Other enzymes may hydrolyze the protein or carbohydrate outer coat of bacteria or viruses to reveal a fragile inner coat. Macrophages are also able to kill intracellular microorganisms by interfering with their vital metabolic processes. Fatty acid extracts from activated macrophages have been shown to be bactericidal (Kochan and Golden, 1974).

The inflammatory macrophage also functions extracellularly by releasing hydrolytic enzymes and other substances. These may be important in killing extracellular microorganisms, generating fever and stimulating production of more macrophages (Dannenberg, 1975). These extracellular enzymes may also be responsible for much of the tissue destruc-

tion seen in chronic inflammatory reactions (Page et al, 1974). The persistence of undigestible material within macrophages stimulates the continuous secretion of substances (Gordon et al, 1974). Recent experiments have demonstrated that attachment of C3b to mouse macrophages caused a dose and time dependent release of selected lysosomal enzymes from the macrophage (Schorlemmer et al, 1975). Macrophages may also passively release their cellular contents including lysosomal enzymes when they die.

III. Role of Macrophage in the Specific Immune Response

The role of the macrophage in the induction of a specific immune response has been extensively studied during the past ten years. Much of the experimental work has been summarized in recent reviews (Unanue, 1972; Gottlieb and Waldman, 1972; Unanue, 1975). Even though the importance of cellular interactions between the macrophage and lymphocytes in eliciting a complete immune response has been repeatedly emphasized, the exact role of the macrophage has not been well defined. Many functions, some contradictory, have been attributed to macrophages and it is not yet clear which are the prime roles the macrophage performs in the in vivo situation. I would like to briefly discuss some of the primary functions involving the immune response presently attributed to macrophages.

The action of the macrophage is necessary to form a complete antibody response to many antigens. Mosier and Coppelson (1968) reported, in 1968, that three cell types were needed for the in vitro induction of a primary antibody response to sheep red blood cells. One of the cells was adherent and the other two were nonadherent. They stated that the vast majority of adherent cells were morphologically and functionally macrophages. Later it was demonstrated that other antigens (Brucella abortus, rabies virus, pneumococcal polysaccharide) also required both a macrophage-like cell and nonadherent lymphoid cells for a primary antibody response (Curley et al, 1974).

The major role of the macrophage in the induction of the immune response appears to be that of antigen presentation to the lymphocytes.

Antigens which are bound to macrophages are highly immunogenic both in vitro and in vivo (Unanue, 1972). This was first shown by Unanue and Askonsas (1968) using a haemocyanin antigen and Mitchison (1969) using a bovine serum albumin (BSA) antigen. The antigen was incubated with peritoneal exudate cells, the cells washed and then injected into normal animals. The antibody response was then measured and compared to that when only free antigen was given. Antigen bound to macrophages was immunogenic in all cases. Further experiments with over 20 different antigens have shown that antigens which were poorly trapped in vivo by macrophages, such as BSA or IgG, were strongly immunogenic when administered as macrophage bound antigen. Larger proteins such as haemocyanin which were readily taken up by tissue macrophages, were not as immunogenic as free antigen when administered in a macrophage bound form (Unanue and Calderon, 1975). When the ability of the antigen to be trapped by the macrophages was disregarded as in a completely in vitro system, it was found that DNP-haemocyanin bound to macrophages was about 1000 fold more immunogenic than when given in soluble form.

Most antigen which is taken up by macrophages is rapidly degraded (Mitchison, 1968; Unanue, 1975). Yet immune recognition of the antigen requires recognition of the conformational antigenic-determinants before extensive catabolism has taken place. Unanue (1975) has suggested two mechanisms by which the antigen could be presented to the lymphocytes before extensive antigen degradation. First, small amounts of antigen may not be immediately interiorized but may remain on the macrophage surface long enough to interact with the lymphocyte. Experiments have shown that though 80 to 95% of haemocyanin taken up by mouse peritoneal macrophages was rapidly catabolized and eliminated from the cell; a small amount of the haemocyanin remained on the cell surface. The membrane bound antigen was immunogenic and could be removed by trypsin or ethylenediamine tetraacetate (EDTA) (Unanue and Cerottini, 1970). Second, small amounts of antigen may also be released from the cell before substantial degradation has occurred. This phenomenon has not been consistently observed with all antigens but does occur with haemocyanin

(Calderon and Unanue, 1974). Unanue and Calderon (1975) believe that this mechanism may be important in the release of antigens from complex material such as bacteria, red blood cells and parasites.

The physical interaction between antigen carrying macrophages and lymphocytes may also be important. Antigen dependent lymphocyte stimulation using inbred guinea pig cells has been shown to be macrophage dependent (Waldron *et al*, 1973). Culture supernatants from antigen pulsed macrophages could not replace the presence of macrophages. This suggested that a close physical contact between lymphocyte and macrophage may be necessary for the induction of the response. Using two inbred guinea pig strains, Rosenthal and Shevach (1973) have demonstrated that in order to produce antigen dependent lymphocyte stimulation in the guinea pig, cooperation between a histocompatible macrophage and lymphocyte was necessary. Antigen bound to allogeneic macrophages did not stimulate lymphocytes and semi-allogeneic macrophages cooperated only 50% as well as syngeneic macrophages. They also reported that in the guinea pig system, the macrophage as well as the lymphocyte must possess the necessary immune response (IR) gene (Shevach and Rosenthal, 1973). To explain these observations, they have proposed that the antigen recognition sites on the T lymphocytes are physically related to the sites of macrophage-lymphocyte interaction. If the macrophage lacks the IR gene for a specific antigen, the antigen's binding site will be physically removed from the macrophage-lymphocyte interaction site.

Lipsky and Rosenthal (1973) have described both an antigen independent and antigen dependent binding of lymphocytes to macrophages. Antigen independent binding took place between macrophages and thymocytes or column purified lymph node lymphocytes of the same species but not necessarily same strain. The binding was reversible and appeared to represent a steady state condition between cellular association and dissociation. The presence of antigen increased the degree of macrophage-lymphocyte binding (Rosenthal and Shevach, 1973). The antigen mediated interactions required both macrophages and lymphocytes from syngeneic animals. Continued interaction resulted in the stimulation of lympho-

cyte DNA synthesis within 72 hours. Rosenthal et al (1975) have proposed a sequence of physical events among macrophage, lymphocyte and antigen which leads to T lymphocyte proliferation. Antigen independent binding between macrophage and lymphocyte would take place first. This would gather the lymphocytes around the macrophage without conveying any type of immune specificity. Antigen dependent binding would then occur if both the macrophage and lymphocyte possessed antigen receptors for the antigen bound to the macrophage.

Other functions besides antigen presentation to lymphocytes have been attributed to macrophages. Because the macrophage can efficiently remove and degrade large amounts of antigen, they are thought to be useful in preventing tolerance by large doses of antigen. The macrophage may also alter the antigen to make it more immunogenic by complexing it with RNA (Fishman et al, 1973). Macrophages with surface bound antigen may act as a focus for attracting T and B cells which could then interact more efficiently (Askonas and Roelants, 1974). Finally, some studies have suggested that macrophages may have a role in regulating the immune response by secreting regulatory molecules which can enhance the response of lymphocytes to mitogens and antigens (Calderon et al, 1975; Wood and Cameron, 1975).

IV. The Role of Macrophages in Viral Infections

The importance of the macrophage in combating viral infections has been demonstrated in numerous studies (reviewed by Mims, 1964; Smith, 1972; Allison, 1974; Silverstein, 1975). Many of the functions of macrophages described in the preceding sections are essential to limiting viral spread and to eliminating infectious virus from the body. The macrophage acts by phagocytosis to remove virus particles from the blood and body spaces (Mims, 1964). This clearance of the virus by macrophages is an important barrier against virus spread to vital target organs (Allison, 1972). Virus particles may also be rendered noninfectious in this manner because many viruses are not able to replicate within the macrophage. After entering the macrophage, many viruses undergo an abortive replicative cycle with no production of infectious

virus. The macrophage may also handle the viral particle as a complex antigen and interact with lymphocytes in the production of antibody and the induction of cell mediated immunity against the virus. Both arms of the immune response have been shown to be important in various viral infections. The formation of antibody-virus complexes also serves to enhance phagocytosis of the viral particle by the macrophage. Studies have shown that suckling mice were protected from death by the virus Cocksackie B-3 when they were passively given diluted anti-Cocksackie antibody and peritoneal macrophages from adult mice (Rager-Zisman and Allison, 1973). Either diluted antibody or peritoneal macrophages given alone did not protect the mice from death.

Although the majority of viruses cannot undergo a productive replicative cycle within the macrophage, a number of viruses can infect the macrophage and produce new virus. Table I summarizes the viruses which are known to replicate within macrophages of the various animals listed.

Age dependent resistance to viral infections has been related in some studies to the ability of the maturing macrophage to abort viral infections. This was first suggested by the study of herpes simplex virus (HSV) infection in mice. Johnson (1964) found that resistance to herpes encephalitis in mice developed with age. When newborn mice were given HSV intraperitoneally (i.p.), they died of encephalitis, whereas, the adult mice were resistant. This age dependent resistance was attributed to the ability of the infected adult macrophages to prevent productive herpes virus replication. Further studies of HSV in mice directly related the protective feature in adult mice to mature macrophages. Passive transfer of adult macrophages protected suckling mice against an intraperitoneal challenge with HSV (Hirsch et al., 1970). Herpes simplex virus was later shown to undergo an abortive infection within adult mouse macrophages. Both viral DNA and protein were produced in the macrophage but the parts were not assembled into virions (Stevens and Cook, 1971).

The ability of the mature macrophage to abort viral replication

TABLE I

VIRUSES WHICH REPLICATE WITHIN MACROPHAGES

Virus	Animal	Reference
Aleutian Disease of Mink	adult mink	Porter et al, 1969
Avian Sarcoma Virus B and C subgroups	embryonic and adult chicken	Gazzolo et al, 1974
Ectromelia Virus	adult mice	Roberts, 1964
Fowlpox virus	adult chicken	Pathak et al, 1974
Friend Virus	adult mice	Levy and Wheelock, 1975
Germiston Virus	newborn and adult mice	Olson et al, 1975
Herpes Simplex Virus	newborn mice	Johnson, 1964 Hirsch et al, 1970 Stevens and Cook, 1971
Influenza Virus	adult mice	Shayegani et al, 1974
Lactic Dehydrogenase Virus	adult mice	Brinton-Darnell et al, 1975
Lymphocytic Choriomeningitis Virus	adult mice	Mims and Subrahmanyam, 1966
Measles Virus	neonatal human	Sullivan et al, 1975
Mengovirus	adult mice	Eustatia et al, 1972
Mouse Hepatitis Virus-3	adult mice (susceptible strains)	Bang and Warwick, 1960 Eustatia et al, 1972
Murine Cytomegalovirus	adult mice	Selgrade and Osborn, 1974
Pichinde Virus	adult hamster	Buchmeier and Gangavalli, 1976
Reovirus-Type 3	adult mice	Eustatia et al, 1972
Vaccinia Virus	adult rabbit and adult mice	Tompkins et al, 1970
Vesicular Stomatitis Virus	adult human adult mice	Edelman and Wheelock, 1967 Eustatia et al, 1972
Wesselbron Virus	newborn mice	Olson et al, 1975
Yellow Fever Virus	adult monkey	Tigertt et al, 1959

and its relation to age-dependent resistance has been studied in other animal-virus systems. The resistance of adult mice to Coxsackie B-3 virus has been attributed to both the abortive replication of the virus within the adult macrophage and the maturing of the humoral immune system (Rager-Zisman and Allison, 1973). The age dependent resistance of mice to Wesselbron virus, a group B togavirus, correlated with the ability of the adult peritoneal macrophages to destroy the virus (Olson et al, 1975). Peritoneal exudate macrophages from 21 day old mice protected suckling mice against an intraperitoneal challenge by Wesselbron virus. The severity of measles infection in children less than one year of age has been recently related to the ability of measles virus to infect neonatal monocytes (Sullivan et al, 1975). Peripheral blood monocytes from human cord blood were more than 10 times as susceptible to infection by measles virus than adult peripheral blood monocytes.

The resistance of mice to intraperitoneal infection by rabies virus is age dependent and indirect evidence suggests that macrophages may be involved. Intraperitoneal injections of India ink, silica, and anti-macrophage serum reduced the resistance of adult mice to i.p. injections of rabies virus (Turner and Ballard, 1976). It was not clear whether rabies virus underwent limited replication in suckling peritoneal macrophages. Rabies virus is thermolabile and could be inactivated under the culture conditions. The titer of rabies virus decreased in cultures of both adult and suckling mice macrophages; however, the decrease in titer was 10 fold more in adult macrophages.

The increased susceptibility of suckling mice to murine cytomegalovirus (MCMV) was partially related to the action of the macrophage (Selgrade and Olson, 1974). Treatment of mice with silica increased the susceptibility of CBA mice to i.p. infection with MCMV and led to increased titers of virus in the livers of the mice. Transfer of adult macrophages increased the resistance of suckling mice to MCMV infection. However, these experiments also revealed that silica treatment of the CBA mice did not render them as susceptible to MCMV as the more

susceptible and untreated C57B1 strain. The transfer studies did not support the theory that the macrophage was the singular leading factor in the resistance to MCMV. Although the growth of MCMV in newborn macrophages was not examined, the growth of MCMV in adult macrophages in both the resistant CBA and more susceptible C57B1 mice displayed a similar level of infection. Examination of the effects of silica dust on Mareks disease virus, another member of the herpes virus group, did not reveal an increased susceptibility to disease as that shown with HSV and MCMV (Higgins and Calnek, 1976).

Strain dependent resistance to virus infections has also been related to the fate of the virus within the macrophage (Allison, 1974). There can be variations in both the strain of the virus and the strain of the animal. The growth of virulent and attenuated virus strains in macrophages was first studied by Bang and Warwick (1960) using Mouse Hepatitis Virus (MHV). They demonstrated that the virulent strain MHV-3 could multiply in mouse macrophages while the avirulent strain MHV-1 did not. Roberts (1964) produced similar findings using two ectromelia virus strains. The highly virulent Hampstead mouse strain of ectromelia virus was about 10 times more infective in mouse peritoneal macrophages than the attenuated Hampstead egg strain. Viral strain differences have also been shown with Avian Sarcoma virus (ASV) (Gazzolo *et al*, 1974). Subgroups B and C of ASV were able to replicate in both embryonic (yolk sac) and adult chicken macrophages whereas subgroups A and D did not replicate. The ability to replicate in the macrophage correlated with morphological and sugar uptake changes in the infected macrophages. However, the authors did not relate the fate of the various ASV subgroups within the macrophage to the transforming ability of the virus in the animal.

A correlation between mouse strain and the fate of the macrophage was also reported by Bang and Warwick (1960) in their study of MHV. Using a virulent strain of MHV-2, they demonstrated that the resistant mouse strain (C3H) demonstrated no macrophage destruction while the susceptible mouse strain (Princeton) was vulnerable to macrophage

destruction by the virus. The susceptibility trait was shown by genetic crosses to be inheritable. Other studies have revealed that a correlation does not always exist between the virus-macrophage interaction and the variation in animal strain susceptibility. Roberts (1964) reported that the difference in mouse strain susceptibility to ectromelia virus was not related to the ability of the virus to replicate within the macrophage. The same held true for MCMV. Although CBA mice were more susceptible to MCMV than C57Bl mice, there was little difference in the growth of the virus within the two strains of peritoneal macrophages (Selgrade and Osborn, 1974). Recent studies with Pichinde virus in hamsters have also revealed no correlation between the ability of Pichinde virus to grow in peritoneal macrophages and the course of the viral infection in LVC and MHA hamsters (Buchmeier and Gangavalli, 1976).

Allison (1974) has suggested that if a virus can multiply within the macrophage but cause no cytopathic effect (CPE) in the animal's cells, this leads to viral persistence. Several persistent or slow viruses have been shown to multiply in the macrophage. Lactate dehydrogenase virus (LDV) causes a life long viremia in mice with no clinical signs of illness (Brinton-Darnell *et al*, 1975). The macrophage appears to be the principle site of LDV replication. Primary macrophage cultures yield more infectious virus than cultures from any other type of mouse tissue. The replication of LDV in the macrophage causes no obvious cytopathic effect (CPE). Lymphocytic choriomeningitis virus (LCMV) is a persistent virus in mice. Macrophages taken from infected mice contain the virus. It was also shown that macrophages taken from LCMV carrier mice are resistant to infection by ECMV whereas normal mouse macrophages are susceptible to LCMV (Mims and Subrahmanyam, 1966). The etiological agent of Aleutian Disease of Mink has been shown to replicate in the macrophages of mink (Porter *et al*, 1969). Immunofluorescence studies of Aleutian disease virus infected mink have found viral antigen in the cytoplasm of splenic and lymph node macrophages and of Kupffer cells.

V. Heterogeneity of Macrophage

Investigators studying the macrophage do not agree on the role of the macrophage in the induction of the immune response and in the elimination of infectious agents and tumor cells. Some investigators feel that the macrophage is only a scavenger cell with the sole function of removing antigen from the system and preventing tolerance due to antigenic overdose. Other investigators believe that the macrophage is capable of enhancing the antigenicity of antigens either by simple attachment to the macrophage surface or by some type of antigen processing. Other investigators feel that the macrophage also releases nutritive factors which provide the necessary environment for responding lymphocytes (Rice and Fishman, 1974). The macrophage is also reported to eliminate tumor cells by both cytostasis and cytolysis. These diverse functions attributed to macrophages make it probable that the macrophage population is not functionally uniform and that the diverse functions are due to a functionally heterogenous population of cells (Walker, 1976).

It is possible to separate peritoneal exudate (PE) macrophages into several populations with varying functions. Using bovine serum albumin gradients, Rice and Fishman (1974) separated rabbit PE cells into three subpopulations of macrophages. One population was highly endocytic but produced no immunogenic RNA. Immunogenic RNA was tested for by incubating the cells with T2 phage, extracting the cellular RNA and then adding it to spleen cell cultures. After 4 to 5 days incubation, the cultures were assayed for neutralizing antibody. Another subpopulation of macrophages displayed little endocytic activity but produced immunogenic RNA that gave rise to both 7s and 19s antibody. Walker (1976) has separated rabbit PE cells on ficoll gradients into subpopulations of cells which yielded immunogenic RNA that induced the production of either IgG or IgM antibody. The cellular subgroups also induced different antigen binding classes of antibody. Walker summarized what is known about heterogenous macrophage populations by stating that it is unclear whether macrophage functional heterogeneity is due to

the degree of cellular maturation, to a differentiation between cells, or to multiple origins of the cells.

VI. The Activated Macrophage - General Description

Confusion surrounds the terminology associated with the activated macrophage. In this thesis, I shall refer to an activated macrophage as a macrophage which has a heightened capacity to destroy intracellular organisms. The term activated macrophage has also been used to describe a macrophage which has undergone certain morphological or biochemical changes or a macrophage which is cytotoxic for tumor cells. Although a correlation among these diverse characteristics has been demonstrated in a few cases, it has not been examined in a majority of the studies reported here. Macrophage activation should not be confused with the induction of cells into the peritoneal cavity by the injection of an irritant such as mineral oil, starch, thioglycollate, or proteose peptone. Macrophages obtained in this manner are normal as regards their ability to destroy intracellular organisms.

George Mackaness (1962) was one of the first to describe activated macrophages. He found that the susceptibility of normal mice to death caused by Listeria monocytogenes was due to the ability of the bacteria to survive and multiply in the mouse macrophage. Convalescent mice, however, were resistant to Listeria reinfection. This was shown to be due to a change in the mouse's macrophages which made them resistant to Listeria. The appearance of the Listeria resistant macrophages correlated with the onset of delayed hypersensitivity. However, the macrophage resistance lasted only three weeks while the hypersensitivity persisted. Mackaness also reported that passive transfer of Listeria immune sera did not protect against Listeria.

Additional studies have shown that resistant macrophages (later termed activated macrophages) may arise during infections by other microorganisms including Toxoplasma gondii (Ruskin and Remington, 1968), Besnoitia (Remington and Merigan, 1969), Brucella (Mackaness, 1964), lymphocytic choriomeningitis virus (Blanden and Mims, 1973), ectromelia virus (Blanden and Mims, 1973), Mycobacteria tuberculosis (Simon and

Sheagren, 1971) and vaccinia virus (Tompkins et al, 1970). In all of these infections, the microorganism can replicate in the normal macrophage but not in the activated macrophage. The resistance of the activated macrophage is immunologically nonspecific and even crosses phylogenetic boundaries (Mackaness, 1964). Many investigators including Mackaness (1964), Ruskin and Remington (1968), and Remington and Merigan (1969) have shown that macrophages activated by a specific bacterium resisted other intracellular bacteria as well as protozoa and viruses. Protozoan activated macrophages were resistant against intracellular bacteria and viruses (Ruskin and Remington, 1968; Remington and Merigan, 1969; Ruskin et al, 1969) and virally activated macrophages resisted viruses as well as intracellular bacteria (Mackaness, 1970; Tompkins et al, 1970; Blanden and Mims, 1973).

The rate of development and duration of the activated macrophage depend on the type of infection. Activated macrophages appear rapidly (4 days) in acute infections where the infecting agent reaches large numbers quickly. Chronic infections such as tuberculosis and brucellosis stimulate a slow rate of macrophage activation (Mackaness, 1964, 1970). The duration of the activated state depends on the persistence of the inducing antigen (Mackaness and Blanden, 1967). Shortly after the infectious agent is cleared from the host, nonspecific macrophage resistance disappears and is followed by the loss of specific macrophage resistance (Mackaness, 1970). Some type of immunological memory is associated with macrophage resistance (Mackaness and Blanden, 1967). Activated macrophages reappear more rapidly during reinfection with the same organism but are induced at a primary rate if reinfected by an unrelated organism.

Several morphological and biochemical characteristics have been associated with in vitro cultures of activated macrophages (Roberts, 1964; Mackaness, 1970). Activated macrophages are reported to be larger than normal macrophages and to contain increased numbers of phase dense lysosomes and cytoplasmic vacuoles. They attach to glass more rapidly and have increased spreading ability. Activated macrophages have also

been reported to exhibit an increased mitotic rate, respiration rate and digestive capacity with a high level of acid hydrolases. Stubbs et al (1973) found that activated macrophages from mouse peritoneal cavities had a normal content of DNA and protein but had an increased glucose oxidation rate. There have been conflicting reports concerning phagocytic rates in activated macrophages. Activated macrophages have been reported to exhibit an enhanced rate of phagocytosis for such particles as dead Mycobacteria (Roberts, 1964) but to exhibit no increase for other particles such as starch and Candida albicans (Ruskin et al, 1969; Stubbs et al, 1973).

VII. Induction of Activated Macrophages

Early studies with activated macrophages suggested a relationship with the immunological delayed hypersensitivity reaction. Both activated macrophages and delayed hypersensitivity arose at approximately the same time in the animal and both could be passively transferred with cells but not serum (Mackanness, 1964). Only living nonadherent spleen cells were effective in transferring the activation of macrophages. Treatment of lymphocytes by sonication, mitomycin C or anti-lymphocyte serum reduced or blocked the induction of activated macrophages (Mackanness, 1970).

In vitro studies have confirmed that the induction of activated macrophages is mediated by lymphocytes (Patterson and Youmans, 1970; Simon and Sheagren, 1971; Krahenbuhl and Remington, 1971; Bast et al, 1974). The experiments were performed by incubating immune spleen cells and the specific sensitizing antigen for 72 hours with a normal macrophage monolayer. The monolayer was then washed to remove all nonadherent cells and the test organism was added. Intracellular growth of the bacteria, protozoa, or virus was then monitored. Only sensitized lymphocytes which had been incubated with their sensitizing antigen were able to mediate changes in the macrophage which led to increased antimicrobial activity.

Further experiments have suggested that it is the T cell or lack of it which is influential in macrophage activation. An early indica-

tion of this was the finding in 1968 by Hirsch *et al* (1968) that mice injected with anti-theta serum (ATS) had increased morbidity and mortality to vaccinia virus. Later studies demonstrated that ATS treated mice were also more susceptible to mousepox virus (Blanden, 1970) and Listeria (Pearson and Osebold, 1973, 1974). Mice made T cell deficient by thymectomy and lethal irradiation were not capable of developing resistance to Listeria (Blanden, and Langman, 1972) or of resisting challenge to a heterologous bacteria (North, 1974). Both specific and non-specific resistance, however, were restored by an infusion of syngeneic thymocytes. Spleen cells treated with ATS plus complement were also incapable of transferring resistance to Listeria (Lane and Unanue, 1972). The surprising finding was that even though spleen cells taken from mice which were adult thymectomized, lethally irradiated, bone marrow reconstituted (ATx-BM) could not transfer protection to Listeria, the ATx-BM mice did not show increased susceptibility to Listeria infection (Cheers and Waller, 1975; Zinkernagel and Blanden, 1975). Two different groups of workers have recently demonstrated that the macrophages of ATx-BM mice are always activated, even before infection (Cheers and Waller, 1975; Zinkernagel and Blanden, 1975). Because of their activated macrophages, the ATx-BM mice are more resistant to infection by Brucella and Listeria than normal controls. The macrophages of congenitally athymic "nude" mice were also shown to be normally in the activated state. Both groups of workers suggested that the activation of macrophages in the absence of T cells could be explained by either of two theories: 1. Bacterial phospholipid extracts are able to nonspecifically activate macrophages (Faune and Hevin, 1974); these bacterial products might leave the gut and nonspecifically activate the macrophages, 2. B cells have recently been shown to produce various lymphokines. One of these B cell factors might be responsible for macrophage activation. A recent report by Meltzer (1976) supports the theory that bacterial products are responsible for macrophage activation in untreated nude mice. Peritoneal macrophages from conventionally raised nude mice were nonspecifically cytotoxic to tumor cells in vitro. Peritoneal macrophages from

nude mice raised under germ free conditions, however, were not tumoricidal in vitro. This suggested that macrophage activation in the nude mouse is dependent on environmental stimuli and is not directly related to the absence of a thymus.

The activation of macrophages by stimulated lymphocytes appears to be mediated by (a) lymphocyte produced soluble factor(s) or lymphokine(s). Such a soluble factor has not yet been isolated from the serum of immune animals, possibly because it circulates at very low concentrations. Blanden and Mims (1973) have suggested that a blood borne factor might be one way of explaining the activation of peritoneal macrophages which are far from the initial site of infection.

In vitro experiments have definitely implicated a soluble factor released from sensitized lymphocytes as the mediator of macrophage activation in culture (Patterson and Youmans, 1970; Krahenbuhl and Remington, 1971; Simon and Sheagren, 1972; Klein and Youmans, 1973; Nathan et al, 1973; Adams et al, 1973; Klein et al, 1973; Bast et al, 1974; Sethi et al, 1975; Borges and Johnson, 1975). The experiments involved culturing sensitized spleen cells with their specific sensitizing antigen for up to 72 hours. The cell free supernatant was then collected and added to a normal macrophage monolayer for an additional 72 hours. Macrophage activation was then assayed for by growth inhibition of intracellular organisms or by morphological or physical methods (amount of adherent cell protein, level of glucose oxidation). The time required for the two incubations often varied among different laboratories. A recent report on the activation of human monocytes by Toxoplasma has even reported the production of an active supernatant after only 15 minutes incubation and the activation of the monocytes after a two hour exposure to the supernatant (Borges and Johnson, 1975). Active supernatants were not produced when sensitized spleen cells were incubated without their sensitizing antigen or when macrophages were incubated with only antigen but no lymphocytes.

Supernatants from nonspecifically stimulated lymphocytes also activate macrophages in vitro. Codal et al (1971) found that either 3

to 4 day or 6 to 7 day old culture fluids from rabbit mixed leucocyte cultures caused proliferation, vacuolation, giant cell formation, and fusion when incubated with a rabbit macrophage monolayer for three days. The intracellular growth of Mycobacteria was also inhibited. No changes were seen in cells cultured with fluid from control leucocyte cultures. Culture fluid from Concanavalin-A stimulated lymphocytes were able to activate both guinea pig and mouse macrophages (Klein and Youmans, 1973; Nath et al, 1973). A macrophage activating factor was also found in the supernatants of phytohemagglutinin (PHA) stimulated spleen cells taken from a mouse which had 12 days earlier received a skin allograft (Caraux et al, 1975). No activating factor was found in PHA stimulated spleen cells from nongrafted mice. The authors felt that this was the first indirect evidence that a macrophage activating factor was produced in response to graft antigens by animals carrying allografts.

Evans and Alexander (1971) have described a soluble factor which renders macrophages cytotoxic to tumor cells. Specific macrophage arming factor (SMAF) is produced by immune T cells when they are cultured in the presence of specific antigen. Macrophages treated with SMAF become armed against the specific inducing antigen. If the macrophages are treated with SMAF plus specific antigen, the macrophages become non-specifically activated. SMAF has been separated by gel chromatography into two molecular weight preparations (Evans et al, 1973). The sizes of SMAF in the mouse are 50,000 to 60,000 daltons and greater than 300,000 daltons (Evans et al, 1972). SMAF has been shown to bind in a specific manner to the target cells used to sensitize the host. These target cells were then killed in the presence of normal macrophages. SMAF will also bind to normal macrophages which then become specifically cytotoxic (Evans et al, 1972; Pels and Den Otter, 1974). The activity of the cytotoxic macrophages could be removed by trypsin treatment.

Macrophage activating factor has been associated with macrophage migration inhibition factor (MIF). Present evidence suggests that macrophage activating factor may be indistinguishable from MIF. David (1975) feels that in all probability macrophage activating factor and

MIF are the same material. MIF rich fractions from lymphocyte culture supernatants have been able to activate macrophages (Nathan *et al.*, 1971; Krahenbuhl and Remington, 1971; Adams *et al.*, 1975; Nath *et al.*, 1973; Nathan *et al.*, 1973). The fractions were usually separated using Sephadex G 100 or G 200. Nathan *et al.* (1973) have performed the most intensive studies on the various lymphokines and have not been able to separate macrophage activating factor from MIF but are able to separate macrophage activating factor from both macrophage chemotactic factor and lymphotoxin. Both macrophage activating factor and MIF eluted from Sephadex G 100 at a peak molecular weight of between 35,000 and 55,000. This molecular weight excluded both antigen-antibody complexes and cytophilic antibody (Dy *et al.*, 1976). Macrophage activating factor also had a significant peak in the region where albumin elutes. Both macrophage activating factor and MIF had a bouyant density in CsCl which was greater than either chemotactic factor or lymphotoxin. The bouyant density of macrophage activating factor was 1.427 to 1.329. Macrophage activating factor and MIF were both sensitive to neuraminidase while chemotactic factor and lymphotoxin were not. The bouyant density and neuraminidase sensitivity of macrophage activating factor and MIF are consistent with a glycoprotein composition. Pronase treatment of mouse macrophages prevented the activation of macrophages if applied before the activating supernatant but not after the addition of macrophage activating factor (Dy *et al.*, 1976). This suggested that macrophage activating factor acts to modify the macrophage rather than to be passively carried by the macrophage as a surface molecule.

Both macrophage activating factor and MIF appear to exert their effects without being consumed by the macrophage (Nathan *et al.*, 1973). This was measured by culturing an active supernatant with macrophages for 3 days with no decrease in the activity of the supernatant. This is in variance with the findings that SMAF coats the macrophage as well as the target cells and disappears from the supernatant after a short period of incubation (Pels and Den Otter, 1974). A further difference between the two activating substances is that SMAF can be removed by

trypsin with the abolition of the activated state.

A variation in the in vitro time course response to macrophage activating factor and MIF was the only dissimilarity between the two found by Nathan et al (1973). Macrophage migration inhibition can be measured during the first 24 hours of culture with macrophages. Macrophage activation will only appear after the macrophage has been cultured for three days with the factor. The authors concluded that macrophage activating factor and MIF are the same factor but that their various effects have different times of response.

There have been three reports of the lack of association between macrophage activating factor and MIF. Klein et al (1973) reported that cultured spleen cell supernatant inhibited the intracellular growth of Mycobacterium tuberculosis without the inhibition of macrophage migration. Different media, however, were used for MIF and macrophage activating factor production and neither method nor data for the measurement of MIF was presented. Simon and Sheagren (1972) were able to detect MIF without macrophage activation. They used lymphocyte supernatant produced by the incubation of spleen cells with antigen for 24 hours and then added it to the macrophages for another 24 hours before assaying for macrophage activation. Other studies have reported that macrophage activation is slower to develop than migration inhibition and generally takes 72 hours. The third report, by Osebold et al (1974), described the induction in mice of enhanced resistance to Listeria without the development of delayed hypersensitivity. A diffusion chamber containing Listeria was placed in the peritoneal cavity of mice. When tested later, some of the mice did not develop delayed hypersensitivity as measured by footpad enlargement 24 hours after challenge. These same mice had decreased mortality when challenged with lethal doses of Listeria. A statistical analysis of the data was performed but the significance in death rates was not readily apparent. One should also note that enlargement of footpads at 24 hours may not be the best method of measuring delayed hypersensitivity in mice.

The roles of interferon and antibody in the increased resistance

of animals to intracellular organisms have been investigated and both interferon and circulating antibody have been ruled out as the cause of macrophage activation. Several interferon inducers given to mice 18 hours before challenge with Listeria or Salmonella typhimurium did not reduce the death rate whereas mice previously infected with Toxoplasma gondii were resistant (Ruskin and Remington, 1968). Remington and Morgan (1969) found that mice persistently infected with either Toxoplasma or Besnoitia were resistant to Mengo virus for up to one year. Interferon was not felt to be a factor because protozoan infections induce interferon production only during their early stages. Humoral antibody was ruled out because the ability to activate macrophages has never been transferred by passive injection of serum from resistant animals (Miki and Mackaness, 1964; Ruskin *et al*, 1969; Remington and Merigan, 1969; Mackaness, 1970). *In vitro* incubation of immune serum with macrophages had no effect on the intracellular growth of Listeria (Miki and Mackaness, 1964).

Although cytophilic antibody has been suggested as a possible mediator of macrophage activation, there is no reported evidence to support this hypothesis. Since macrophage activation cannot be passively transferred with serum (Miki and Mackaness, 1964; Mackaness, 1970), the cytophilic antibody would have to attach very avidly to the macrophages and would have to be present in a free state only in very small amounts. Macrophages have a surface receptor for the Fc portion of an antibody molecule and can bind antibody *in vivo*. It is possible to elute this cell associated antibody from macrophages by gently heating. Ueda and Nozima (1973) were able to elute anti-vaccinia neutralizing antibody from the peritoneal macrophages taken from vaccinia virus sensitized mice. Cell bound cytophilic antibody could possibly enhance the macrophage binding of specific antigen but this does not explain the nonspecific effect of activated macrophages. Tizard (1971) has hypothesized that cell bound antibody complexed with antigen may activate the macrophage's enzyme systems which would lead to enhanced killing ability against any intracellular organism. Few experiments have been performed

to examine this or any other role cytophilic antibody may have with the induction of activated macrophages.

Macrophages may be activated by both immunological and nonimmunological methods. As described earlier, activated macrophages may be obtained from animals after infection with a wide variety of microorganisms. Activated macrophages are also produced after passive injection of sensitized syngeneic lymphocytes plus their sensitizing antigen. It is possible to produce activated macrophages in vitro by treating them with lymphocyte supernatants produced either during an immunological reaction such as lymphocytes reacting with their sensitizing antigen or a mixed leucocyte reaction or during a nonimmunological reaction such as treatment with concanavalin A. Activated macrophages can also be produced in a nonspecific manner by treating cells either in vivo or in vitro with endotoxin, lipid A or double stranded RNA (Alexander and Evans, 1971). In vitro treatment with a low molecular weight peptoglycan isolated from Mycobacteria will also activate macrophages.

VIII. Activated Macrophages and Viruses

The activated macrophage has been studied in relation to only a few viral systems. These limited studies have confirmed that macrophages activated in a viral system possess similar characteristics to those in a bacterial or protozoan system. Influenza virus has the capacity to replicate in the peritoneal macrophages of normal mice but not in macrophages taken from mice immunized with influenza virus either intraperitoneally or nasally (Shayegani et al, 1974). Nonspecific activation was demonstrated by immunizing the mice with Staphylococcus aureus and then challenging the peritoneal exudate cells in vitro with influenza virus. The production of influenza virus specific antigen was markedly lower in the bacterially immunized cells than in the normal cells; however, it was slightly higher than in the virally immunized cells. Similar results were obtained with Fowlpox virus in chicken peritoneal exudate macrophages (Pathak et al, 1974). Peritoneal macrophages taken from chickens infected with Fowlpox virus 15 to 20 days previously were resistant to infection by Fowlpox virus as well as New-

castle Disease virus and the bacteria Salmonella gallinarum.

The interaction between virus and activated macrophage has been most intensively studied with vaccinia virus. Vaccinia virus can replicate in normal PE-macrophages of rabbits (Tompkins et al, 1970; Avila et al, 1972) and mice (Ueda and Nozima, 1973; Koszinowski et al, 1975) but not in PE-macrophages taken from vaccinia virus immune animals. Avila et al (1972) reported that vaccinia virus activated rabbit macrophages demonstrated vaccinia virus specificity and were not refractile to challenge by myxoma virus. I do not believe this to be a general phenomenon and suggest that it may be due to the time the cells were taken after the initial infection with vaccinia virus (4 to 6 weeks after the first inoculation and 1 to 2 weeks after the second). Titering of infectious virus has shown that vaccinia virus adsorbed equally well to activated and normal macrophages (Avila et al, 1972). Activated macrophages did not lose their immunity to vaccinia virus when extensively washed (10 times) with Hanks Balanced Salt Solution or after trypsin treatment (0.25%) for 20 minutes at 37°C (Schultz et al, 1974). IgG displaying very weak neutralization to vaccinia virus could be eluted from the activated macrophages by heating at 37°C. This elution did not alter the activation of the macrophages. Activated macrophages did lose their immunity to vaccinia virus when they were cultured for 7 days at 37°C. Koszinowski et al (1975) working with vaccinia virus infected mouse PE macrophages has suggested that the presence of IgG anti-vaccinia antibody on the surface of activated macrophages is in some cases responsible for the increased clearance from the medium of free vaccinia virus. The clearance of vaccinia virus from the medium by macrophages taken from mice injected 3 times in 14 days with vaccinia virus could be blocked with anti-mouse IgG. Similar treatment of cells taken from mice injected only once in 6 days did not block viral clearance.

IX. Vaccinia Virus

Vaccinia virus is the type species of the viral group Poxvirus. It is a member of the subgroup vaccinia of which cowpox, ectromelia,

variola, rabbitpox, and alastrim are also members (Joklik, 1966). Vaccinia virus is a large irregular shaped virus approximately 270 nm by 218 nm (Moss, 1974). Electron micrographs of thin sections of vaccinia virus reveal a well defined outer membrane which surrounds a dumbbell shaped core and two oval shaped lateral bodies which lie on either side of the core (Joklik, 1966). The outer membrane is lipoprotein in nature and unlike other viral envelopes, is synthesized de novo by the virus (Fenner et al, 1974). The viral core consists of a protein membrane surrounding the virion DNA and internal proteins. The virion nucleic acid is double stranded DNA which may be cross linked at both ends (Geshelin and Berns, 1974). The molecular weight of the DNA has been estimated to be about 150×10^6 (Joklik, 1968b). This corresponds to a coding potential of around 160 proteins with a molecular weight of 50,000 each. The vaccinia virus core also contains several viral enzymes: a DNA dependent RNA polymerase, a polyA polymerase, a nucleoside triphosphate phosphohydrolase, a DNase and a protein kinase (Moss, 1974).

The replicative cycle of vaccinia virus is thought to take place entirely in the cellular cytoplasm although recent findings suggest that some viral DNA may be synthesized in the cell nucleus (La Colla and Weissbach, 1975). Most of the newly produced vaccinia virions remain cell associated and under laboratory conditions are released only after mechanical disruption of the cell. Vaccinia virus infection causes cellular cytopathic effect (CPE) (Joklik, 1966). The initial CPE, after 1-1/2 to 2 hours of infection, is cell rounding and is most extensive after infection with high multiplicities. The second type of CPE consists of cell fusion and becomes evident after the onset of viral DNA synthesis.

The first step in the initiation of vaccinia virus infection, is the adsorption of the virus to the cell. Adsorption of virus to the cell is rapid and is dependent on Mg^{++} ions (Joklik, 1964). The virion enters the cell by the process of cellular phagocytosis (Moss, 1974). Within the phagosome, the outer membrane is rapidly removed and the

viral core is released into the cellular cytoplasm (Joklik, 1968b). This first stage of uncoating is not prevented by inhibition of RNA or protein synthesis. The second stage of uncoating involves the release of the viral DNA and is prevented by inhibition of RNA and protein synthesis or by ultraviolet inactivation. It takes place after a 1/2 to 2 hour lag period which is dependent on the multiplicity of infection (Joklik, 1964). It appears that during this lag period, the core associated RNA polymerase transcribes a message off the core enclosed viral DNA for a viral uncoating protein which is necessary for complete vaccinia virus uncoating (Kates and McAuslan, 1967a). The extent of vaccinia virus uncoating is never 100% and is probably closer to 50% (Joklik, 1964).

The production of vaccinia virus RNA is divided into early and late phases. The early synthesis of viral RNA occurs during the first 2 hours of infection and is probably made entirely in the infecting virion cores (Moss, 1974). The amount of RNA produced is dependent on multiplicity of infection and cell type used. The early RNA produced is primarily 10s to 14s and includes information from approximately 14% of the viral genome. The rate of early RNA synthesis declines as viral DNA synthesis begins and progeny DNA is released from the cores. The burst of late RNA synthesis takes place 3 to 6 hours after infection and is dependent on viral DNA synthesis. The entire genome is transcribed from the progeny DNA. Both early and late viral mRNA contain poly(A) of 100 to 200 nucleotides in length (Nevins and Joklik, 1975).

Vaccinia virus DNA synthesis begins 2 hours after infection and is complete by 6 hours after infection when virion formation begins (Joklik and Becker, 1964). The shut off of viral DNA synthesis appears to be under some type of protein control because it is not related to the disappearance of template or the depletion of precursor molecules. Continuous vaccinia virus protein synthesis is necessary for viral DNA synthesis (Joklik and Becker, 1964; Kates and McAuslan, 1967b).

The synthesis of early and late vaccinia viral proteins is under transcriptional control. The early and late proteins, however, cannot

be divided into enzymatic or structural functions. Structural proteins are formed both before and after the onset of DNA synthesis (Moss, 1974). Thirty different structural polypeptides have been identified by SDS-polyacrylamide gel electrophoresis. Vaccinia induced nonvirion enzymes are also synthesized. These include thymidine kinase, DNA polymerase, several DNases, and a polynucleotide ligase (Fenner *et al*, 1974). Several viral proteins undergo posttranslational modification. At least three major proteins are cleaved from precursor proteins (Katz and Moss, 1970a; Pennington, 1973; Moss and Rosenblum, 1973). Other proteins are glycosylated or phosphorylated (Moss *et al*, 1971; Pogo *et al*, 1975).

Vaccinia virus morphogenesis has been studied most extensively using the electron microscope. At 2 to 3 hours after infection, granular or fibrous dense areas appear in the cytoplasm (Morgan, 1976). These have been called viral factories and are the site of viral component synthesis and assembly. At 3 hours, arcs and circles of membrane begin to appear in the factories. Some of the circles contain small dense nucleoids which are the viral DNA molecules. Virion cores form from these nucleoids. With the addition of the lateral bodies and the outer limiting membrane, the maturing virion migrates away from the factory into the cytoplasm where most of them remain until cell lysis.

X. Purpose of Study

Although it has been known for some time that macrophages play an important role in the defense against viral infections (Mitchison, 1969), the mechanisms by which macrophages limit virus replication have not been examined. The limitation of virus replication in other cell types has been shown to be mediated by interferon; however, a role for interferon in triggering the interference to virus replication in macrophages has not been demonstrated. The present study was, therefore, undertaken to characterize the abortive infection of vaccinia virus in activated macrophages and to compare the findings with those expected if the abortive infection were inhibited through interferon.

MATERIALS AND METHODS

I. Cells

A continuous line of African green monkey kidney cells (Vero) was used for growth and titration of vaccinia virus. The cells were grown in Eagle's modified Minimal Essential Medium (MEM) supplemented with 10% heat inactivated fetal calf serum, 10mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 10 mM hydroxyethylpiperazine N-2 ethansulfonic acid (Hepes), ph 7.2, and 0.15% NaCO₃.

Primary rabbit kidney cells were prepared by overnight trypsinization with 0.5% trypsin at 4°C of small pieces of kidney taken from 2 to 3 week old rabbits. The cells were seeded in tissue culture flasks and grown to confluency in MEM medium as described above.

Rabbit peritoneal exudate (PE) cells were harvested from the peritoneal cavity 3 days after an intraperitoneal injection of approximately 40 ml of sterile mineral oil. The rabbits were bled by cardiac puncture and then killed with an injection of sodium pentobarbital. The skin lying over the peritoneal cavity was cut away and an incision was made in the peritoneal wall. The peritoneal cavity was washed out with several rinses of HBSS. The PE cells were then washed twice with cold HBSS by centrifugation at 12,000 rpm for 10 minutes each at 4C. A sample of the cells was diluted in 0.1% trypan blue and placed in a hemacytometer for counting. Only the large and medium sized cells which excluded trypan blue were counted.

Peritoneal exudate macrophages were activated in vivo by infecting normal rabbits with vaccinia virus (Avila et al, 1972). The rabbits were injected with a total of about 1×10^7 PFU of vaccinia virus stock in a total of six sites on their shaven backs using a 26 gauge needle. Vaccinia lesions first appeared 2 days later as red areas. Small vesicles formed at the injection sites by 4 to 5 days after infection.

II. Animals

Young adult New Zealand white rabbits of both sexes were obtained locally and used throughout the experiments. They were housed in individual cages and given food and water ad libitum.

III. Vaccinia Virus: Preparation of Stocks and Titration of Virus

Virus stocks of the WR strain of vaccinia virus were grown in primary rabbit kidney cells (PRK) or Vero cells. The PRK cells yielded a virus stock which titered between 2 to 7×10^7 PFU/ml while a virus stock prepared in Vero cells contained about one half log less virus. Confluent monolayers of cells were infected with approximately 0.1 PFU/cell of virus. The virus was allowed to adsorb for one hour at room temperature and MEM plus 2% FCS was added. The cultures were incubated at 37°C for two days after which the cells were then freeze-thawed twice to liberate the virus. Cell debris was removed by low speed centrifugation and the virus containing supernatant was dispensed in small vials and stored at -50°C. Tritiated thymidine labelled vaccinia virus was produced by the addition of 1 μ c/ml of 3 H thymidine (20 ci/mole, New England Nuclear) to the medium after virus adsorption.

Virus titrations were performed on confluent monolayers of Vero cells grown in 60 mm dishes. Ten fold dilutions of the virus suspensions were made in cold Hanks Balanced Salt Solution (HBSS). Two tenths ml of the diluted virus was pipetted onto the Vero cells and allowed to adsorb for 45 minutes at room temperature. Four ml of MEM + 2% FCS was then added to each plate. The plates were incubated at 37°C in 5% CO₂ for 2 to 3 days until the virus plaques were visible. The media was then removed and FAA fixative (see Appendix I) was placed on the cells for 1 to 5 minutes. This was poured off and the monolayer was stained with 1% crystal violet in water. The excess stain was rinsed off with water and the plaques were counted.

IV. Virus Purification

Vaccinia virus was purified according to a method described by Joklik (1962) and modified by Katz and Moss (1970b). Virus infected cells and culture medium were frozen and thawed to lyse the cells. The

cell debris was then removed by centrifugation at 1500 rpm for 10 minutes. The debris was resuspended in a small amount of the supernatant and sonicated for 30 seconds at full power in the cup of a Bronwill Biosonik IV sonicator to further release the virus. The debris was again sedimented and all of the supernatants were pooled. Virus in the supernatant was pelleted through a 36% sucrose cushion by layering in a 30 ml Oakridge tube 25 ml of supernatant over 5 ml of 36% sucrose in 0.01 M Tris, pH 9. This was spun at 25,000 x g for 60 minutes. The supernatant was carefully removed and the pellet resuspended in 0.5 ml of 0.01 M Tris, pH 9. The resuspended pellet was then layered on a 25% to 40% continuous sucrose gradient (0.01 M Tris, pH 9) and spun at 14,000 x g for one hour. Forty drop fractions were collected by bottom puncture and the optical densities at 260 nm and at 280 nm were recorded. The fractions containing the virus peak were pooled and diluted in 0.01 M Tris, pH 9. The virus was then pelleted by spinning at 25,000 x g for one hour and resuspended in a small amount of buffer. The last two centrifugations were repeated to obtain very pure virus for use in antibody production. To estimate the amount of purified virus obtained, the following formula was used: $1.00 \text{ OD unit}_{260} = 1.2 \times 10^{10} \text{ elementary bodies/ml}$ (Joklik and Becker, 1964).

V. One Step Growth Curves of Vaccinia Virus

Activated and normal rabbit peritoneal macrophages were collected and washed twice in HBSS. The cells were counted and 2.4×10^7 cells were resuspended in a small volume (approximately 0.4 ml) of HBSS. One ml of vaccinia virus stock was added to give a multiplicity of infection (MOI) of 2. This mixture was incubated on ice for 30 minutes with frequent shaking. After the adsorption period, unadsorbed virus was removed by adding 50 ml of HBSS to the tube and pelleting the cells at 1000 rpm for 10 minutes. The HBSS was poured off and the cells were resuspended in MEM + 10% FCS to give a cell concentration of 10^6 cells/ml. One ml aliquots of cell suspension were placed in culture tubes and incubated in a slant position at 37°C in a 5% CO₂ incubator. At the sample times, duplicate tubes were frozen at -50 until ready for assay.

Cell samples were freeze-thawed twice to release intracellular virus before titration.

The virus growth curve in Vero cells was performed in a similar manner. Confluent monolayers of Vero cells were trypsinized with 0.25% trypsin-EDTA to remove the cells. The cells were then washed, counted, and resuspended in a small volume of HBSS to which vaccinia virus was added at a MOI of about 2. The medium control consisted of mixing 4×10^7 PFU of vaccinia virus with 25 ml of MEM + 10% FCS, distributing one ml aliquots into tubes and incubating them. Duplicate tubes were frozen and assayed in parallel with the cell cultures. The duplicate tubes from each time sample were pooled and assayed on Vero cells.

VI. Adsorption of Vaccinia Virus to Rabbit Macrophages

Forty million PE cells or 2×10^7 Vero cells were suspended in 4.5 ml of HBSS and placed in an ice bath. Five one hundredths ml of purified vaccinia virus labelled with ^3H thymidine (about 22,000 CPM, 7.5×10^8 PFU) was added to the cells and the mixture quickly dispensed in 0.3 ml aliquots into tubes sitting in a rocking ice bath. The tubes were continually rocked in the ice bath until the sampling time when 20 ml of cold PBS was added to triplicate tubes to stop the adsorption. The cells were washed by spinning at 12,000 rpm for 10 minutes. One ml of the supernatant was counted by mixing with 10 ml of scintillation fluid containing 10% (Biosolv, Beckman) BBS-3. The pellet was dissolved in 0.5 ml of 20% BBS-3 in water and then counted in 10 ml of scintillation fluid containing 20% BBS-3. The mean number of counts in the pellet, in the supernatant, and in the total tube were calculated from the triplicate tubes at each sample time. The results were expressed as a percentage of the number of counts present in the pellet over the counts present in the entire tube.

VII. Elution of Virus from Cells and Virus Uncoating

The experiments to study the elution of vaccinia virus adsorbed to cells were performed in conjunction with the vaccinia virus uncoating experiments. Forty million PE cells or 2×10^7 Vero cells were mixed with 0.05 ml of purified vaccinia virus labelled with ^3H -thymidine

(25,000 CPM). The cell-virus mixture was divided into 6 tubes and the virus allowed to adsorb for 30 minutes in a rocking ice bath. Unadsorbed virus was removed by washing with cold phosphate buffered saline (PBS) and the cells were resuspended in two ml of MEM + 10% FCS. The tubes were incubated at 37°C. At hourly sample periods, the cells were pelleted and a one ml sample of the supernatant was mixed with 10 ml of scintillation fluid containing 10% BBS-3 and counted. The susceptibility of the eluted virus to the activity of DNase was tested by adding 0.15 ml of DNase (0.5 mg/ml) to one ml of supernatant, incubating for 30 minutes and then precipitating with 0.2 ml of 50% trichloroacetic acid (TCA). The cell pellet was used to assess vaccinia virus uncoating.

The cell pellets were resuspended in 1 ml of .01 M NaPO_4 , .01 M MgCl_2 , pH 7.0 buffer, and frozen and thawed twice. The samples were divided into two 0.5 ml portions. One portion received 0.1 ml of DNase (0.5 mg/ml) while the other portion received 0.1 ml of the .01 M NaPO_4 MgCl_2 buffer. The tubes were incubated for 30 minutes at 37°C and the amount of digested DNA determined by precipitating each sample with 0.2 ml of 50% trichloroacetic acid (TCA) and then counting a sample of the supernatant and the TCA precipitate. The percentage of specific ^3H thymidine counts released into the supernatant by the DNase was calculated by subtracting the number of counts in the buffer supernatant from the counts in the DNase supernatant and then making a percentage of the total counts in each tube.

VIII. Virus DNA Synthesis

Vaccinia-virus DNA synthesis was studied by pulse-labelling at various times after infection with ^3H thymidine and then assaying for the appearance of TCA precipitable counts. The procedure involved seeding 5×10^6 PE cells in 35 mm cell culture dishes and incubating for one hour at 37°C. The cells were then infected with either 0.5 ml of vaccinia virus stock (7×10^7 PFU/ml) or 0.5 ml of MEM + 2% FCS and incubated for 30 minutes. The inoculum was removed and 0.5 ml of MEM + 2% FCS was added to the dishes which were again placed at 37°C. At hourly intervals, the cells were pulsed for 5 minutes with 5 $\mu\text{C}/\text{ml}$ of ^3H thymi-

dine. After the 5 minute labelling period, the medium was removed and the cells were scraped into one ml of cold reticulocyte standard buffer (RSB) and allowed to swell for 10 minutes on ice. The cells were then ruptured by ten strokes of a Dounce homogenizer. The nuclear and cytoplasmic fractions were separated by spinning at 800 x g for 5 minutes. The nuclear and cytoplasmic fractions were then separately precipitated by the addition of one ml of 15% cold TCA. The precipitates were collected and counted using scintillation fluid containing 10% BBS-3.

The synthesis of vaccinia DNA in Vero cells was examined in a similar manner. Confluent monolayers of Vero cells in 60 mm dishes were infected with one ml of vaccinia virus (7×10^7 PFU/ml). The cells were pulsed with 2.5 μ C/ml of 3 H thymidine. Only the cytoplasmic fraction was TCA precipitated and counted.

IX. Analysis of Vaccinia Virus Infected Cells by Polyacrylamide Gel Electrophoresis

Seventy-five million PE cells were infected with vaccinia virus at a MOI of 2.5 for 30 minutes on ice. The cells were then washed and resuspended in 12 ml of HBSS. Four ml aliquots of the cells were placed in 60 mm cell culture dishes and incubated for 30 minutes at 37°C to allow the macrophages to adhere to the plates. The medium and any non-adherent cells were then removed and 4 ml of MEM containing 1/10 amino acids were added to the plates.

To detect vaccinia virus specific polypeptides synthesized at 0, 2, 4, 6, and 8 hours after infection, the medium was removed at the sample time and 2 ml of amino acid free MEM containing 25 μ C/ml of 3 H amino acids (New England Nuclear) was added to the plates. The cells were then incubated for 30 minutes at 37°. After the labelling period, the medium was removed and the cells were solubilized in 0.5 ml of slab gel sample preparation buffer and then frozen. For the pulse-chase experiments, all the cells were pulsed with 25 μ C/ml of 3 H amino acids for 30 minutes after 6 hours of incubation with virus. The 3 H containing medium was then removed and 4 ml of MEM plus 10% FCS was added to the plates which were incubated for various chase periods. The cells were

then solubilized and frozen as described above.

Polvacrylamide slab gels containing sodium dodecyl sulfate (SDS) were prepared and run according to the procedure of Laemmli (1970). The gel consisted of a 5% stacking region and a 12.5% separating region. The gel was run in an apparatus containing Tris-glycine buffer plus 0.1% SDS. Dissolved samples were boiled for 2 to 3 minutes and ten lambda of bromphenol blue was added to 0.1 ml of the cooled sample which was then applied to the gel. The gels were usually run at 100 volts for approximately 15 hours at 23°C. After running, the gels were immediately placed in 0.25% Coomassie Blue slab gel stain and stained for 2 to 3 hours at room temperature. They were then destained in several changes of slab gel destain until all nonspecific stain was removed.

Gels containing ^3H or ^{14}C labelled samples were prepared for fluorography by the procedure of Bonner and Laskev. (1974). Basically this involved soaking the gel in two changes of dimethyl sulfoxide (DMSO) for 30 to 60 minutes each and then placing the gel in a solution of 18% 2,5 diphenyloxazole (PPO) in DMSO for 3 hours. The gel was then rinsed in water for 1 to 2 hours and dried onto a piece of filter paper using a Bio Rad Gel Slab Dryer. The dried gel was placed on a sheet of RP-X-Omat film (Kodak) which had been preexposed in a darkroom (for 10 to 15 seconds) by placing it 10 inches from a red safelight. The film and gel were held together in a press and stored at -70°C for 4 to 14 days. The films were developed in an automatic x-ray film developing machine.

X. Virus Particle Production

The production of vaccinia virus particles was assessed by replicating the virus in the presence of radiolabelled precursors of protein or DNA. The distribution of the radiolabel in continuous sucrose gradients was compared with that of marker vaccinia virus. About 9×10^7 rabbit PE cells were infected with vaccinia virus at an MOI of 2.5. After 30 minutes at 4°C, the cells were washed with 50 ml of HBSS and resuspended in 20 ml of HBSS. The cell suspension was equally distributed into two 100 mm plastic cell culture dishes. The dishes were incubated at 37°C in a 5% CO_2 incubator for 45 minutes to allow the

macrophages to adhere to the dish. The medium and any nonadherent cells were then removed and 5 ml of the appropriate medium containing radioactive label was added to the dish. For labelling of proteins, medium consisted of Eagles salt solution plus one tenth the normal amount of amino acids to which 1 μ c/ml of 14 C amino acid mixture (New England Nuclear) was added. DNA was labelled using MEM+2% FCS to which 5 μ c/ml of 3 H thymidine was added. The cells were incubated at 37°C for either 8 hours or 22 hours and the plates were then frozen at -50°C until used.

Vero cells in 100 mm plastic culture dishes were infected with 1.25×10^7 PFU of vaccinia virus in 0.5 ml. After adsorption for 30 minutes, 5 ml of the appropriate medium was added and the plates were incubated at 37°C for either 8 or 22 hours at which time they were frozen.

The frozen plates containing the radioactively labelled infected cells were thawed and the medium plus cell debris was transferred to a 12 ml conical centrifuge tube. The cell debris was removed by spinning at 15,000 rpm for 10 minutes and the supernatant was placed in a 30 ml Oakridge tube. Approximately 20 ml of 0.01 M Tris, pH 9 was added to the tubes which were then spun at 18,000 rpm for one hour to pellet any viral particles. Each pellet was resuspended in 0.4 ml of 0.01 M Tris, pH 9 plus 1% FCS. Twenty five lambda of purified 3 H thymidine labelled vaccinia virus (virus marker) was added to the resuspended pellets labelled with 14 C amino acids. The resuspended pellets were then placed on 25% to 40% sucrose gradients and spun at 12,000 rpm for one hour. Twenty five drop fractions were collected by bottom puncture. In most experiments, the entire fraction was mixed with 10 ml of scintillation fluid containing 5% BBS-3 and then counted. For the experiment in which vaccinia virus antigens were detected by complement fixation, 0.025 ml of each fraction was removed for counting of radioactivity.

XI. Immunological Procedures

A. Antisera

Antiserum to vaccinia virus was raised in rabbits. This was performed by initially infecting rabbits subcutaneously (SC) in the back

with 3×10^7 PFU vaccinia virus and then by repeated intravenous (i.v.) injections of purified vaccinia virus. The initial i.v. injection was given on day 13 and it contained 5×10^8 particles while subsequent i.v. injections were given on days 20, 27, 55, 74 and 113. The subsequent injections consisted of 5×10^8 , 5×10^8 , 4×10^9 , 5×10^{10} and 2.5×10^{10} particles respectively. Samples of serum were collected prior to each injection and the animals were exsanguinated 18 days after the final injection. Antibodies were detected by immunodiffusion using lysates of virus infected cells as antigen. Three rabbits were immunized and the serum which produced 8 immunodiffusion bands against virus antigen and no bands against control cell antigen was selected for use in the experiments.

B. Immunodiffusion

Antigen used for immunodiffusion was prepared by seeding approximately 2×10^7 activated or normal PE cells in a 60 mm plastic cell culture dish and incubating one hour until the cells formed a confluent monolayer. One ml of vaccinia virus (3.5×10^7 PFU/ml) was allowed to adsorb to the cells for one hour at 37°C . One ml of MEM with either 2% NRS or 2% FCS was then added to the cells which were incubated at 37°C . At various times the medium was removed from one plate and the cells were scraped into 0.5 ml normal saline. The cells were frozen and thawed and then sonicated before use as an immunodiffusion antigen.

Microscope slides used for immunodiffusion were precoated with agar by placing one ml of 0.5% agarose (Sigma, electrophoresis grade) in water on each slide and allowing the agarose to dry to a thin film. A thin layer of 1% agarose in borate buffered saline (BBS), pH 8 was applied by taping both ends of the precoated slide with 3 layers of marking tape. A clean slide lightly coated with silicone grease was placed on the taped slide and hot agar was pipetted between the two slides to completely fill the space. After the agar hardened, the plain slide was removed and a plastic template was placed on the agar surface. The template wells were filled with the test antigens and the antiserum. Any air bubbles were removed with a small needle. The slides were then

placed in a humidified chamber and left at room temperature for 3 days. The wells were refilled after the first day if the level of sample had dropped. After 3 days, the slides were prepared for staining by removing the template and rinsing in PBS for one day with frequent changes of the PBS. The slides were then air dried and stained for 15 minutes in Coomassie Blue stain for immunodiffusion slides and destained 10 to 15 minutes in immunodiffusion destain buffer.

C. Indirect Fixed Immunofluorescence

For immunofluorescence, cells were mixed with 2.5 PFU vaccinia virus per cell and the virus was adsorbed for 30 minutes. Unadsorbed virus was removed by washing with HBSS and the cells were resuspended in MEM + 10% normal rabbit serum at a concentration of 1×10^6 cells/ml. Aliquots of cell suspension were placed in sterile 20 mm shell vials which contained an 18 mm round coverslip. These were then incubated at 37° with 5% CO₂. At intervals, the coverslips were rinsed in three changes of PBS for five minutes each. The coverslips were air dried, acetone fixed for 10 minutes and again air dried. The staining procedure involved wetting the coverslips with PBS and placing two drops of a 1/100 dilution of the rabbit serum on the coverslip which was then incubated at 37°C for 30 minutes in a humidified incubator. After incubation, the coverslips were rinsed twice with PBS. A drop of a 1/40 dilution of the fluorescein conjugated IgG fractionated from goat antirabbit gamma globulin (Cappel Lab.) was placed on the coverslips which were again incubated for 30 minutes at 37°C in a humidified incubator. The coverslips were then thoroughly rinsed in PBS and mounted in Tris buffered glycerol, pH 9. The coverslips were examined using a Leitz Ortholux microscope with BG 38 and KP 490 excitor filters and a K 530 barrier filter. Photographs were made on Kodak High Speed Ektachrome Film (Tungsten) using exposures of two minutes.

XII. Interferon Studies

Interferon was prepared in two ways. Tissue culture interferon was prepared from a secondary culture of rabbit kidney cells and was a gift from Dr. Jan Desmyter. The interferon had been induced by infect-

ing secondary rabbit kidney cells with Newcastle Disease virus (NDV) and then harvesting the culture medium at 24 and 48 hours. The culture fluid had been lowered to pH 2.0 and incubated for 6 days to inactivate all the NDV, clarified by centrifugation and then restored to pH 7 with NaOH. Poly (I)-Poly (C) induced serum interferon was produced in an adult rabbit by the intravenous injection of 8 mg of Poly(I)-Poly(C) (MILES). Five hours later, the rabbit was sedated with Nebutal and bled. The serum was removed from the clotted blood, filtered through a 0.45 micron filter and frozen in small vials at -50°C .

The interferon preparations were titrated in rabbit kidney (RK) cells using vaccinia virus and vesicular stomatitis virus (VSV) as challenge viruses and in normal rabbit PE cells using vaccinia virus. Primary rabbit kidney cells were prepared as described earlier and secondary cultures of these cells were seeded into 35 mm dishes and grown until confluency. One ml of the interferon preparation diluted in MEM + 2% FCS was added to the RK monolayers and incubated overnight at 37°C . The cells were then washed twice with warm HBSS and infected with either 0.1 ml of vaccinia virus (8×10^6 PFU) or 0.1 ml of VSV (3×10^7 PFU). The virus infected plates were incubated one hour at 37°C and the unadsorbed virus removed by three washes with warm HBSS. One ml of MEM + 2% FCS was then added to each plate. Three plates were immediately frozen to give a zero time value and the remaining plates incubated overnight and then frozen. Vaccinia virus was assayed on Vero cells and VSV was assayed on BHK-21 monolayers using an agar overlay medium.

Interferon titrations with normal rabbit PE cells were performed in a similar manner except in cell culture tubes. One million freshly harvested and washed PE cells were seeded in each cell culture tube and then incubated overnight with the appropriate interferon dilution. The cells were washed with warm HBSS by centrifugation and then infected with 0.1 ml of vaccinia virus (8×10^6 PFU) for one hour. The cells were then washed three more times with HBSS and then one ml of MEM + 2% FCS was added to each tube. The tubes were incubated overnight and then frozen. Triplicate tubes for each interferon dilution were pooled and

the vaccinia virus titer was assayed on Vero cells.

XIII. The Action of Peritoneal Cell Lysates on Vaccinia Virus

Normal and activated peritoneal exudate cells were collected and washed by the usual procedure. A 20% suspension by volume of each cell type was made in HBSS. The cells were lysed by freeze-thawing and the cell lysate clarified by low speed centrifugation. Equal volumes of cell lysate and vaccinia virus were then mixed to give a final cell lysate concentration of 10%. One tenth ml aliquots of the virus lysate mixture were distributed into small tubes which were incubated at 37°C. Duplicate tubes were then frozen after 0, 1, 3, and 6 hours of incubation. The amount of infectious virus remaining at each time period was titrated by pooling the duplicate tubes and then assaying the virus on Vero cells. A virus stability control was performed by incubating the virus with HBSS in place of the cell lysate.

RESULTS

I. Replication of Vaccinia Virus

The replication of vaccinia virus in normal and activated PE cells was compared with the replication of the virus in Vero cells. The results of a typical experiment are shown in Figure 1. The cell associated virus detected after adsorption was about the same for activated and normal PE cells. This was 3 to 4 times less than the amount which adsorbed to Vero cells. The appearance of progeny virus was detected 7 hours after infection of Vero cells and 9 hours after infection of normal PE cells. No progeny virus was detected in activated PE cells. As can be seen, the virus was relatively thermostable under the conditions of the experiment.

The above experiments were performed using a multiplicity of about 2 PFU/cell. To determine if the interference could be influenced by virus dose, the replication of vaccinia virus in normal and activated PE cells infected with different concentrations of virus was examined. The results shown in Figure 2 indicate that significant virus replication occurred in normal PE cells but not in activated macrophages at multiplicities of infection from 0.1 to 20 PFU/cell.

II. Adsorption and Elution of Vaccinia Virus

The inability of vaccinia virus to replicate in activated macrophages could be due to the inability of the virus to adsorb to the cells or be due to the tendency of the virus to elute from the cells prior to penetration. To test these possibilities, the initial interaction between vaccinia virus and PE cells was examined. An experimental protocol was developed using Vero cells and small aliquots of ³H labelled vaccinia virus. The purified virus and cells were mixed, distributed into separate tubes, and at various times the cell-associated radioactivity was determined. The results of such an experiment with Vero cells are presented in Figure 3. They show that it is possible to mea-

Figure 1

One step growth curves of vaccinia virus. Freshly harvested and washed rabbit PE cells or Vero cells were infected with vaccinia virus at a multiplicity of 2 PFU/cell in an ice bath with frequent shaking for 30 minutes. The cells were then washed and resuspended in MEM + 10% FCS at a concentration of 10^6 cells/ml. One ml aliquots were placed in culture tubes and incubated at 37°C until sampled. Cell samples were freeze-thawed and virus titers were assayed on Vero cells. Normal PE cells (□—□); activated PE cells (●---●); Vero cells (△---△); and mock infected medium (▲—▲).

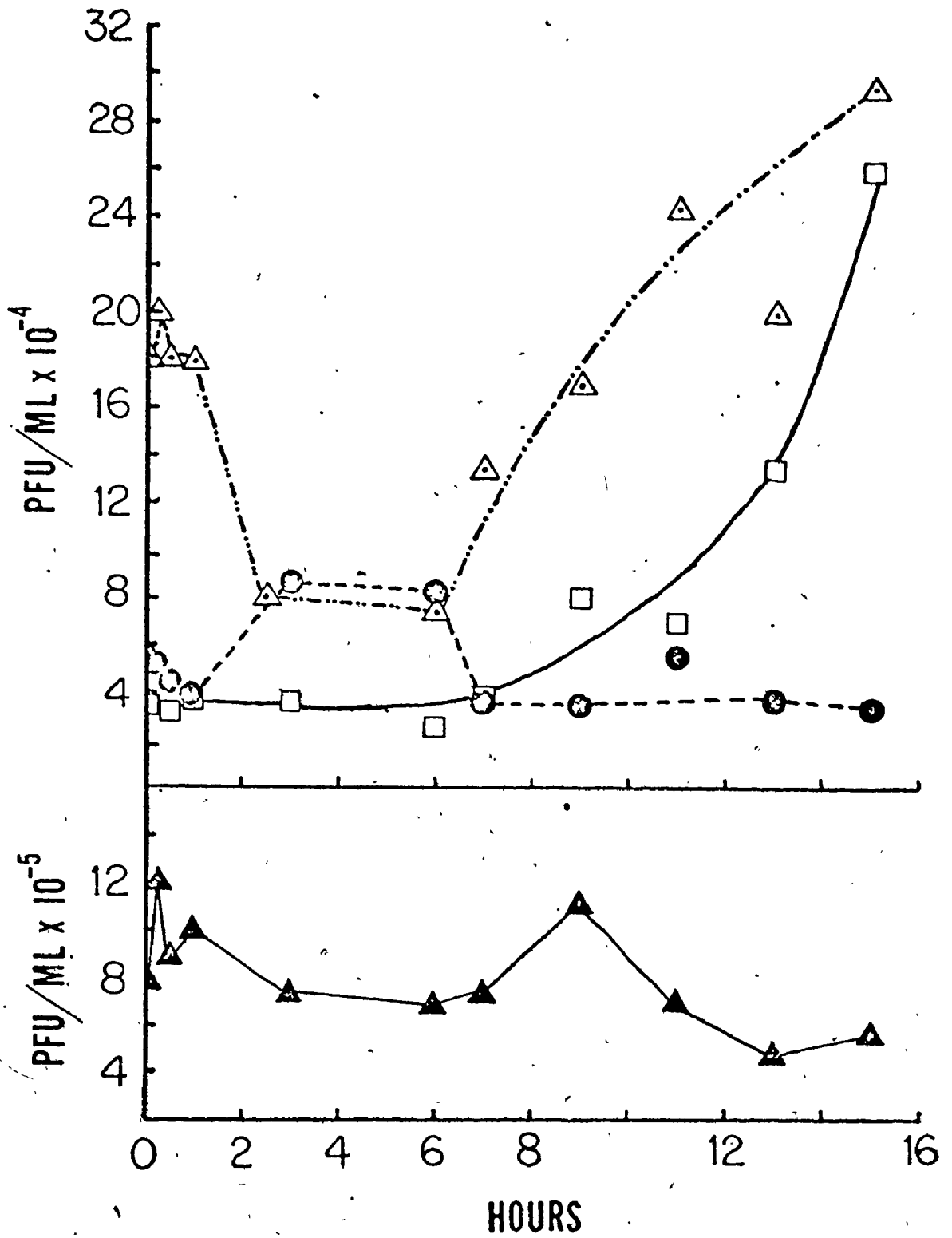


Figure 2

Growth of vaccinia virus in rabbit PE cells. Rabbit PE cells were infected with vaccinia virus at multiplicities of 0.1 PFU/cell (\triangle --- \triangle), 1.0 PFU/cell (\square — \square), 10 PFU/cell (\circ --- \circ) and 20 PFU/cell (\bullet \bullet). After adsorption for 30 minutes in an ice bath, the cells were washed and resuspended in MEM + 10% FCS at a concentration of 10^6 cells/ml. One ml of cell suspension was placed in culture tubes which were then incubated at 37°C. At 24 hour intervals, duplicate tubes were frozen and the virus titered using Vero cells.

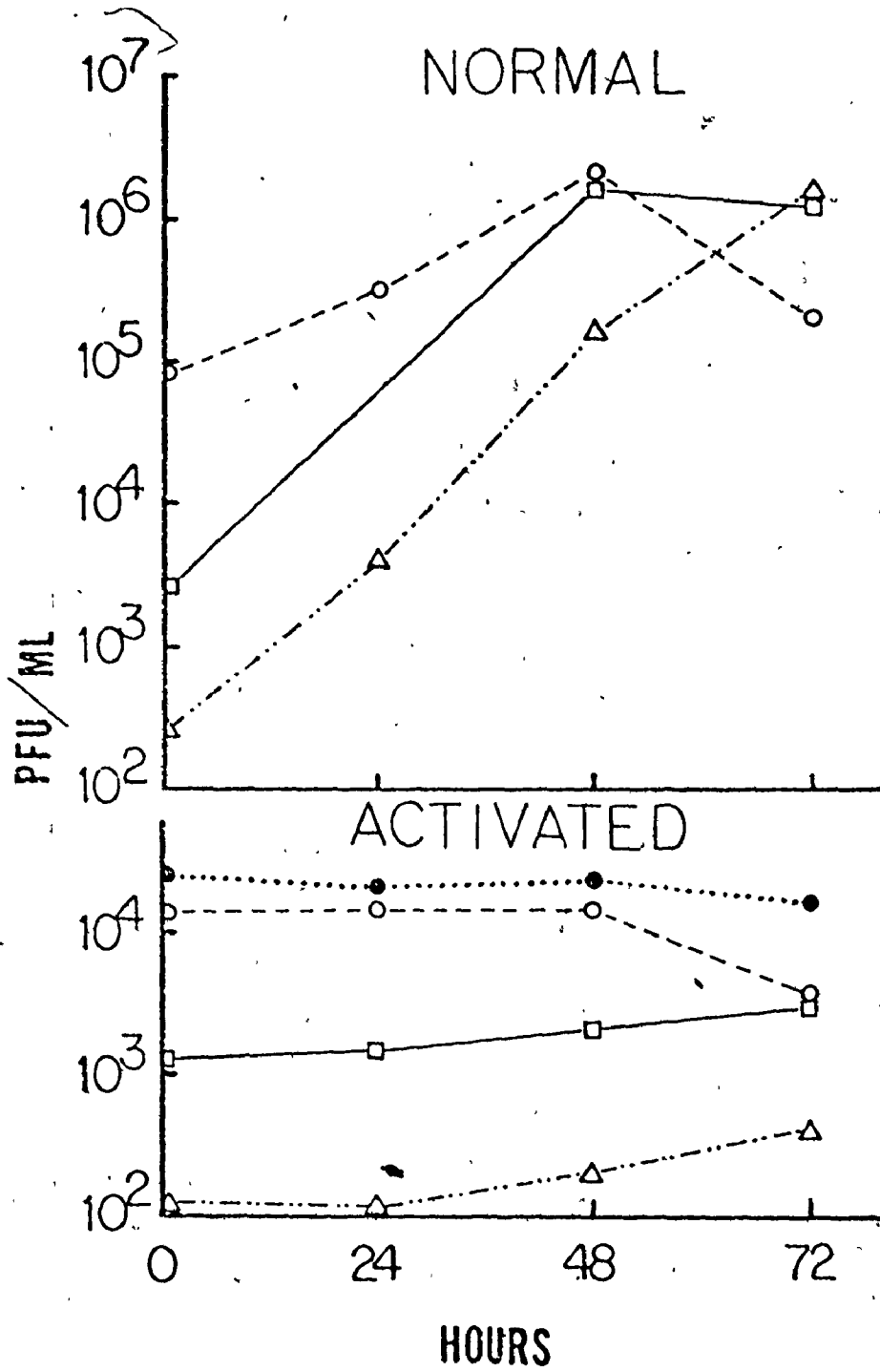
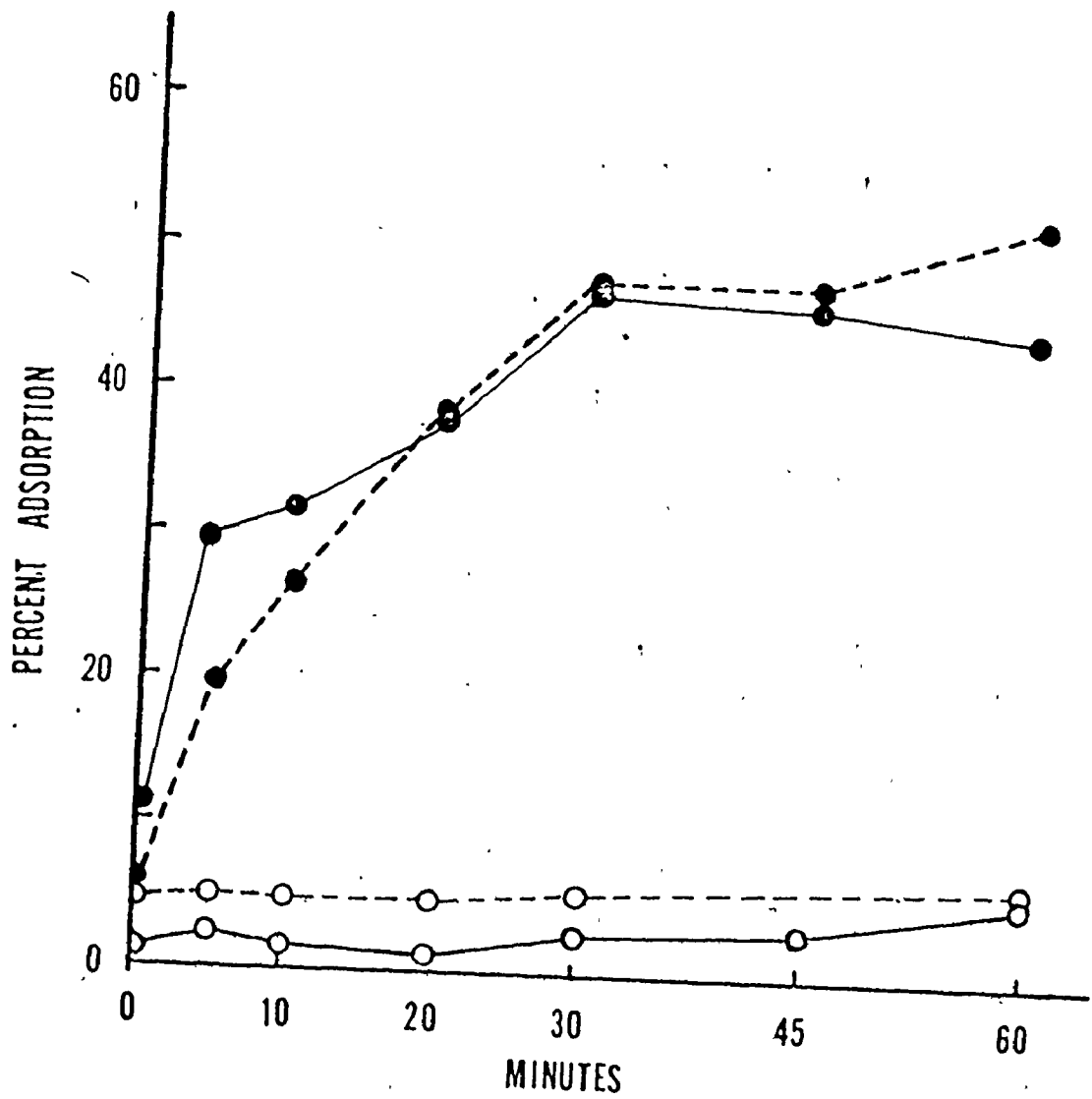


Figure 3

Adsorption of vaccinia virus to Vero cells. Fifty lambda of ^3H thymidine labelled purified vaccinia virus (7.5×10^8 PFU/ml, 23,628 CPM) was mixed with 10^7 Vero cells and immediately distributed into 7 tubes which had been placed in a rocking ice bath. At various times, the cells were washed with 20 ml of cold PBS and samples of the supernatant and cell pellet were assayed for radioactivity. The results were expressed as the percentage of tritium label which was cell associated over the total number of counts in each tube. Vero cells (●---●); PBS control (○—○). The results of two experiments are shown.



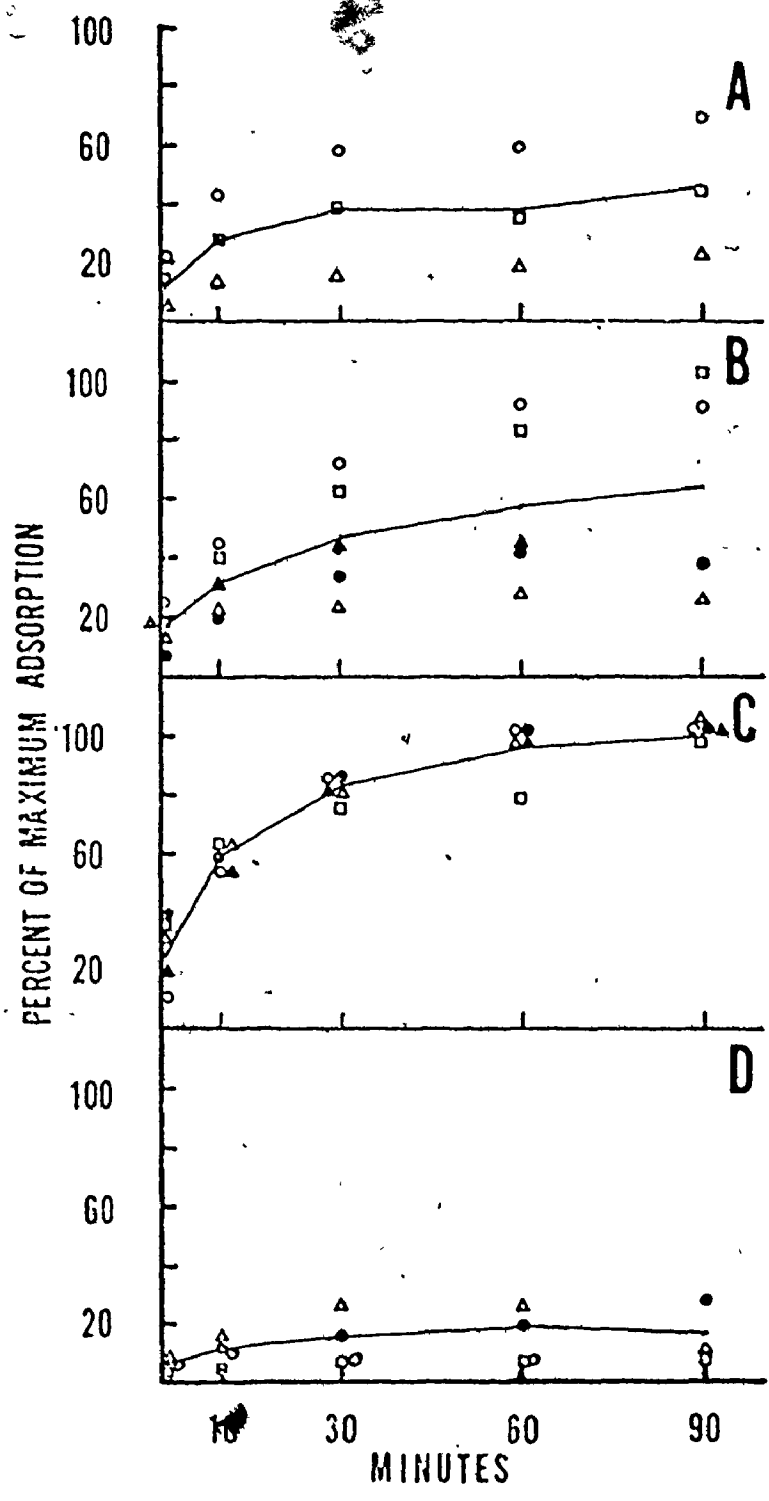
sure adsorption of purified vaccinia virus to cells using ^3H labelled virus and that the maximum amount of adsorption takes place during the first 30 minutes of incubation.

Application of the protocol to PE cells demonstrated that vaccinia virus was capable of adsorbing to activated PE cells even though no infectious progeny was produced. The means of 5 experiments performed with triplicate tubes at each time period are shown in Figure 4. Cells and ^3H thymidine labelled virus were mixed at a MOI of 1 in HBSS and incubated in individual tubes in a rocking ice bath. At the sample time, the cells were pelleted and the amount of ^3H in the pellet and in the supernatant were measured. The results of each experiment were adjusted to a percentage of the maximum level of adsorption for that experiment. In all cases, Vero cells after 90 minutes incubation gave the highest level of adsorption. The actual amount of vaccinia virus adsorbed to the cells was between 10% to 35% of the vaccinia virus available to the cells. The largest increase in adsorption took place during the first 10 minutes of incubation. Adsorption continued for the next 15 minutes with a general leveling off in the amount of adsorption from 30 to 60 minutes of incubation. Vero cells adsorbed almost twice as much vaccinia virus as the PE cells. Activated and normal PE cells adsorbed almost the same amount of vaccinia virus during the first ten minutes of incubation. Vaccinia virus continued to adsorb to the activated PE cells during the next 80 minutes while the amount attached to the normal PE cells leveled off. After 90 minutes of incubation at 0°C , the activated PE cells had 64.5% of the level of Vero adsorption compared to 39% for the normal macrophages. The amount of vaccinia virus sticking to a tube containing only HBSS rose gradually in a linear fashion during the 90 minute incubation period to 24% of the level of Vero adsorption by 90 minutes.

While performing preliminary experiments to detect DNA synthesis in vaccinia virus infected Vero cells, it was noticed that a significant proportion of the virus which had been adsorbed to the cells eluted from the cells during incubation. To compare elution of virus from activated

Figure 4

Adsorption of vaccinia virus to rabbit PE cells and Vero cells. Fifty lambda of ^3H thymidine labelled, purified vaccinia virus (7.5×10^8 PFU/ml, 23,628 CPM) was mixed with 4×10^7 PE cells or 2×10^7 Vero cells and immediately distributed into 15 tubes which had been placed on a rocking ice bath. At various times, triplicate tubes were washed with cold PBS and a sample of the supernatant and cell pellet assayed for radioactivity. The results were adjusted to a percentage of the maximum level of adsorption for each experiment. The results and mean for each cell type is shown. Normal PE cells (**A**); activated PE cells (**B**); Vero cells (**C**); HBSS control (**D**).



macrophages and from normal macrophages, ^3H thymidine labelled virus was adsorbed to the cells at 0°C . Warm MEM + 10% FCS was then added to the cells which were incubated at 37°C until sampled for the amount of virus eluted. The results were expressed as the percentage of virus which had eluted over the total amount of virus initially associated with the cells. The means of 4 experiments are presented in Figure 5. The dissociation curves for activated and normal PE cells are similar. Twenty-six to 30% of the total virus was dissociated from the cells immediately after being resuspended in fresh, warm, serum containing medium. Maximum elution took place during the first hour of incubation, with virus continually dissociating from the cells during the entire 5 hour incubation period. After 5 hours, approximately 55% of the virus which had initially adsorbed to the activated and normal PE cells, had become dissociated from the cells. Vaccinia virus eluted from Vero cells at a much lower rate. Seventeen percent of the virus was dissociated immediately after resuspension in medium. With continuing incubation, viral elution gradually increased to a high of approximately 27% after 5 hours.

A sample of the eluate was treated with DNase in order to determine if the dissociated virus was altered. The results are summarized in Table 2. A large portion of the eluted vaccinia virus was susceptible to DNase. After one hour of incubation, 58% of the virus which eluted from normal PE cells, 63% of the virus which eluted from activated PE cells and 40% of the virus which eluted from Vero cells had become susceptible to the action of the enzyme.

III. Eclipse Phase

The data presented above demonstrated no essential differences in the adsorption and elution of vaccinia virus between normal and activated PE cells. Experiments were therefore undertaken to determine if the virus penetrated and uncoated equally well in the two cell types. An indirect measure of uncoating is the decrease in infectious virus early after adsorption. Tubes containing 1×10^6 cells which had been infected on ice for 30 minutes with vaccinia virus at a MOI of 2 were washed and then incubated at 37°C . The beginning of the incubation

Figure 5

Elution of vaccinia virus from rabbit PE cells and Vero cells. One hundred lambda of ^3H thymidine labelled, purified vaccinia virus ($\sim 100,000$ CPM) was adsorbed to 4×10^7 PE cells or 2×10^7 Vero cells for 30 minutes in a rocking ice bath. The mixture was divided into 6 tubes, the cells washed in PBS and then resuspended in warm MEM + 10% FCS. The cells were incubated at 37°C and, at hourly intervals, the cells were pelleted and the amount of tritium which was cell associated in the supernatant was determined. The means and standard deviations of 4 experiments are represented. Normal PE cells (●---●); activated PE cells (○—○); Vero cells (▲—▲).

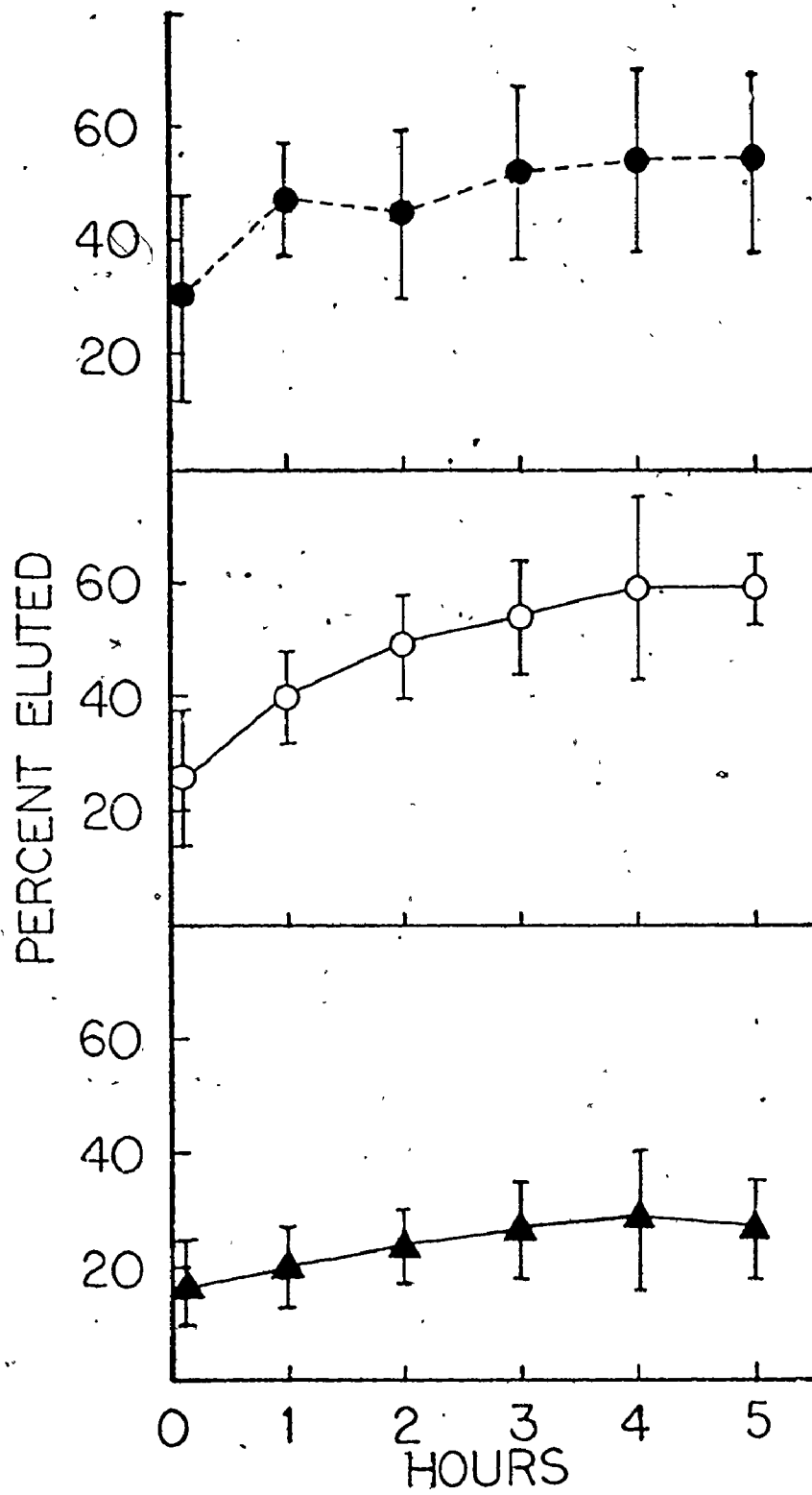


TABLE II

DNase SUSCEPTIBILITY OF ELUTED VACCINIA VIRUS

Time (hrs)	% of ³ H Thymidine Not Acid Precipitable		
	Normal PE Cells	Activated PE Cells	Vero Cells
0	22	25	24
1	58	63	40
2	63	72	49
3	66	73	53
4	68	73	58
5	68	74	58

period was considered zero time. Duplicate tubes were frozen at 15 or 30 minute intervals and assayed for vaccinia virus. The cells from 9 activated rabbits and 9 normal rabbits were examined. In all 9 cases of activated PE cells, the titer of vaccinia virus dropped from one quarter to one half log during the first 2-1/2 hours of incubation. Further incubation provided no additional drop in titer. Vaccinia virus displayed a similar drop in titer in 6 of the 9 experiments using normal PE cells with 3 of the experiments showing no change in vaccinia virus titer during the first 3 hours of incubation. A medium control was performed by incubating vaccinia virus in MEM plus 10% FCS only. Two of the 5 experiments showed a slight rise in vaccinia virus titer during the first hour of incubation while the titer of vaccinia virus remained stationary in the other 3 experiments.

The uncoating of vaccinia virus in the three cell types was studied more precisely by infecting with ^3H thymidine labelled virus and then determining the rate at which the viral DNA became susceptible to DNase. Purified virus was adsorbed to the cells at 4°C and after washing away unadsorbed virus, the cells were incubated at 37°C . At intervals, aliquots of cells were diluted in $0.01\text{ M NaPO}_4 \cdot \text{MgCl}_2$ buffer and submitted to two cycles of freezing and thawing. DNase in quantities shown to be sufficient to digest 100% of a control adenovirus DNA was added and the amount of the cell-associated vaccinia virus DNA susceptible to digestion was determined.

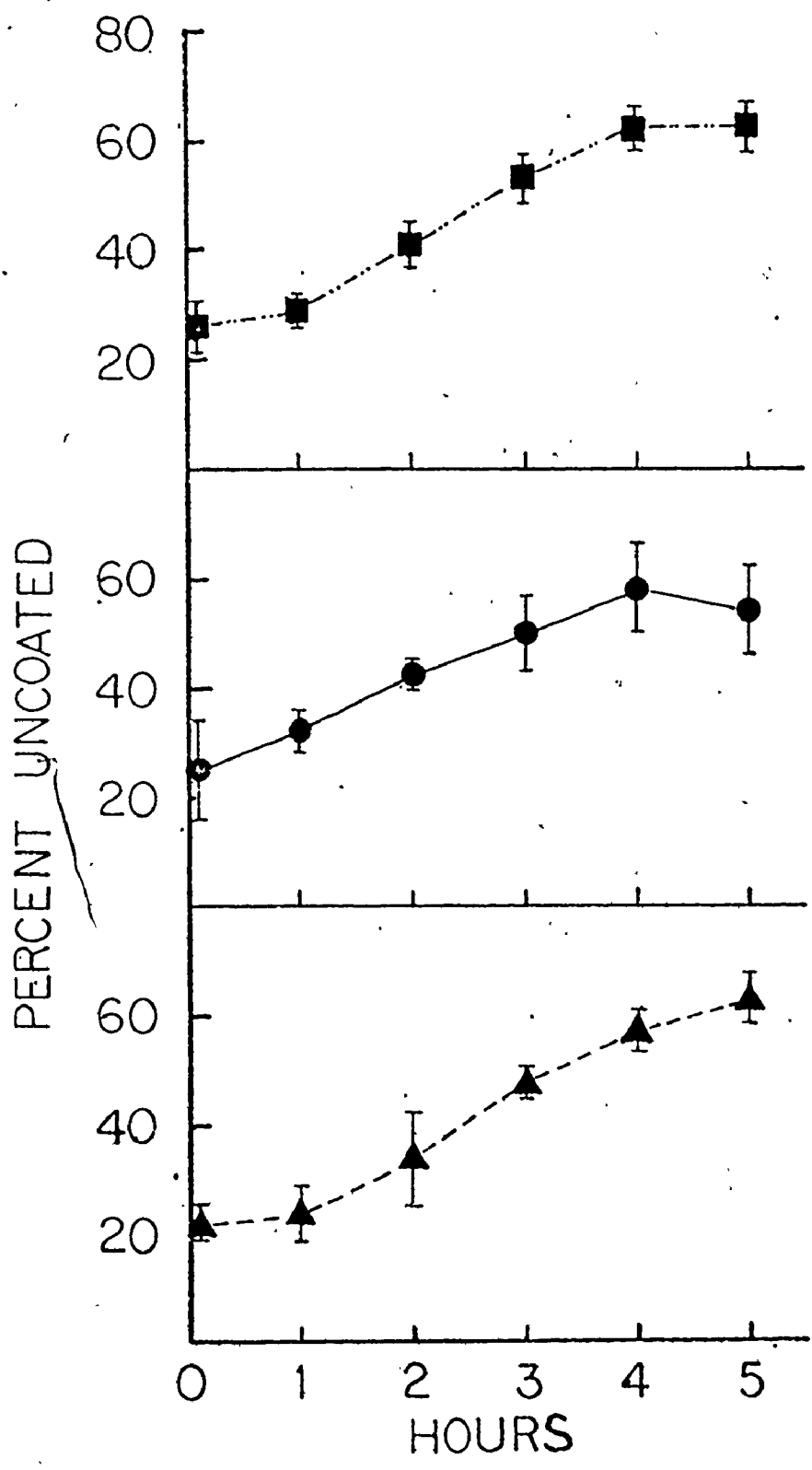
The means of four separate experiments in the uncoating of vaccinia virus in activated and normal macrophages and in Vero cells are plotted in Figure 6. The results are given in terms of percent specific uncoating. All three cell types gave similar uncoating curves. There was a one hour lag before an accelerated rate of uncoating began. The maximum amount of uncoating took place one to four hours after adsorption. The percentage of vaccinia virus uncoated after five hours was approximately 55% in all three cell types.

IV. Virus DNA Synthesis in PE Cells

To determine whether activated macrophages prohibited the replication of vaccinia virus nucleic acid, the synthesis of DNA, as assessed

Figure 6

Uncoating of vaccinia virus in rabbit PE cells and Vero cells. One hundred lambda of purified, ^3H thymidine labelled vaccinia virus ($\sim 100,000$ CPM) was adsorbed to 4×10^7 PE cells or 2×10^7 Vero cells for 30 minutes in a rocking ice bath. The mixture was divided into 6 tubes, the cells washed in PBS and then resuspended in warm MEM + 10% FCS. The cells were incubated at 37°C and, at hourly intervals, the cells were pelleted and resuspended in 1 ml of $0.01\text{ M NaPO}_4 \cdot \text{MgCl}_2$ buffer. The cells were disrupted by 2 cycles of freezing and thawing and then were divided into two tubes. One tube received 0.1 ml of DNase solution (0.5 mg/ml) and the other tube received 0.1 ml of buffer. The tubes were incubated for 30 minutes at 37°C , 0.2 ml of 50% trichloroacetic acid (TCA) was added and the amount of tritium in the TCA pellet and supernatant determined. The percent specific uncoating was calculated by subtracting the number of counts in the buffer treated supernatant from the DNase treated supernatant and dividing that by the total number of counts. The means and standard deviations of 4 experiments are presented. Normal PE cells (■---■); activated PE cells (●—●); Vero cells (▲---▲).



by the incorporation of ^3H thymidine, was monitored in normal and activated PE cells. Preliminary experiments with Vero cells indicated that it was possible to detect a burst of viral DNA synthesis in vaccinia virus infected cells when the TCA precipitable ^3H thymidine counts from the cytoplasmic fractions of infected and noninfected cells were compared (Figure 7). DNA synthesis in the virus infected cells began two hours after infection and increased to a peak at five hours after infection and then dropped rapidly.

Identical experiments on peritoneal macrophages using only the cytoplasmic fraction also showed a greater amount of DNA synthesized in the virus infected cells than in noninfected cells. The difference, however, between the infected and noninfected cells was not as great as in the Vero cells and the burst period of DNA synthesis was not as distinct. It was found that a large portion of the DNA synthesized in the virus infected macrophages was in the pellet following cell fractionation. Therefore, both the supernatant and pellet fractions after Dounce homogenization were counted and plotted separately as well as together for total TCA precipitable counts. In normal macrophages infected with vaccinia virus, an increase in DNA synthesis in the cytoplasmic fraction was obtained two hours after infection. The increase, however, was not great and did not yield a smooth pattern (Figure 8). The nuclear fraction showed a more definite cycle of DNA synthesis in the infected normal macrophages (Figure 9). There was a large burst of DNA synthesis at 3 hours after infection which continued for 3 more hours and then declined rapidly. The levels of DNA synthesis in noninfected cells were constantly low both in the supernatant and pellet fractions. The graph of total TCA precipitable counts also shows a period of peak DNA synthesis from 3 to 6 hours after infection in the normal macrophages infected with the virus (Figure 10). Activated peritoneal macrophages infected with vaccinia virus displayed similar patterns of DNA synthesis in the various fractions.

V. Vaccinia Virus Protein Synthesis

The data presented above suggest that viral DNA synthesis occurs

Figure 7

DNA synthesis in the cytoplasmic fraction of vaccinia virus infected Vero cells. Confluent monolayers of Vero cells (60 mm culture dishes) were infected with 7×10^7 PFU of vaccinia virus for 30 min at 37°C. The virus was removed, 1 ml of MEM + 2% FCS was added and the cells were incubated at 37°C. At hourly intervals, the cells were pulsed for 5 minutes with 2.5 μ C/ml of 3 H thymidine. The cells were then washed with RSB, scraped into 1 ml of fresh RSB and allowed to swell for 10 minutes in an ice bath. The cells were lysed with 6 strokes of a Dounce homogenizer and the nuclear and cytoplasmic fractions separated by spinning at 800 x g for 5 minutes. The cytoplasmic fraction was precipitated with 15% TCA and counted. Vaccinia virus infected Vero cells (○—○); mock infected Vero cells (△—△). These data are representative of two experiments.

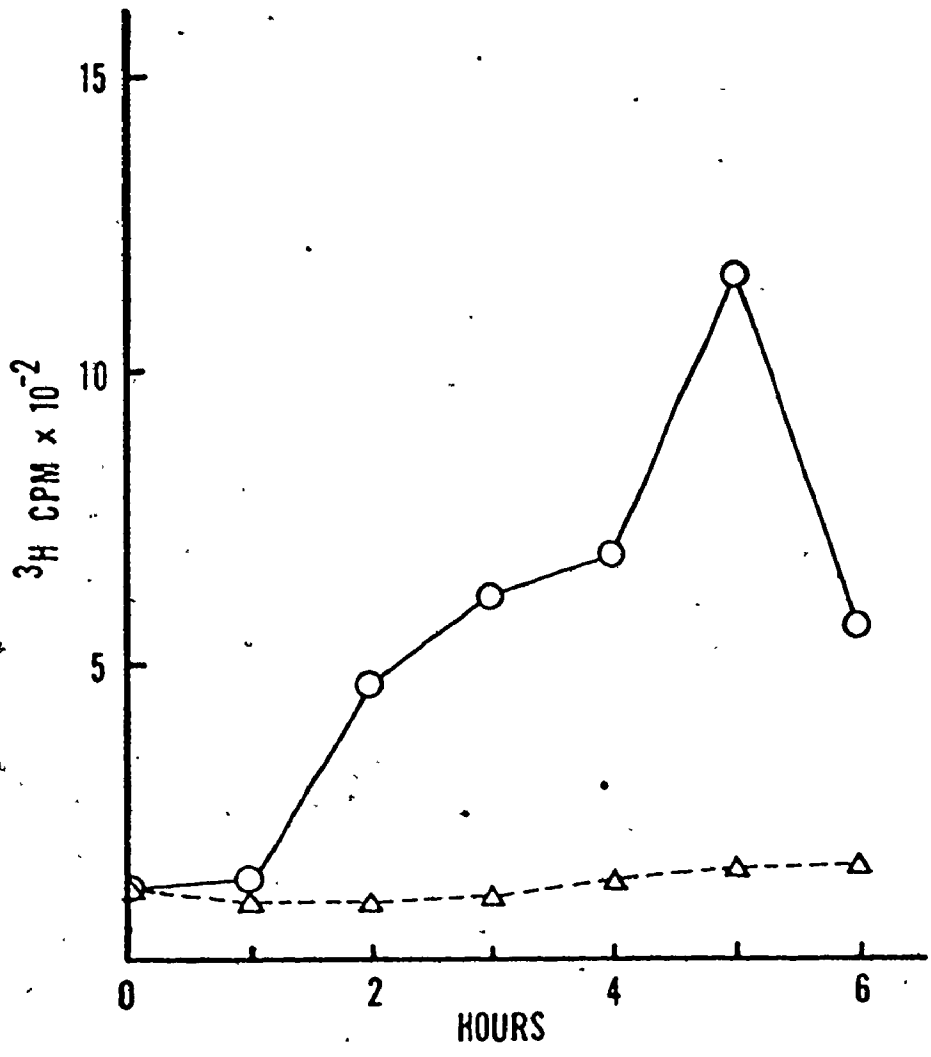


Figure 8

DNA synthesis in the cytoplasmic fraction of vaccinia virus infected PE cells. Confluent monolayers of adherent rabbit PE cells (35 mm culture dish) were infected with 3.5×10^7 PFU of vaccinia virus for 30 minutes at 37°C. The virus was removed and 0.5 ml of MEM + 2% FCS was added to the plates which were incubated at 37°C. At hourly intervals, the cells were pulsed for 5 minutes with 5 μ c/ml of 3 H thymidine. The medium was removed and the cells were scraped into 1 ml of cold RSB and allowed to swell for 10 minutes in an ice bath. The cells were ruptured by 10 strokes of a Dounce homogenizer and the nuclear and cytoplasmic fractions separated by spinning at 800 x g for 5 minutes. The cytoplasmic fraction was precipitated with 15% TCA and counted. Normal PE cells infected with vaccinia virus (●—●); normal PE cells mock infected (○—○); activated PE cells infected with vaccinia virus (▲—▲); activated PE cells mock infected (△—△). These data represent the mean of two experiments. They are representative of the data obtained from two other experiments performed for 6 hours of incubation.

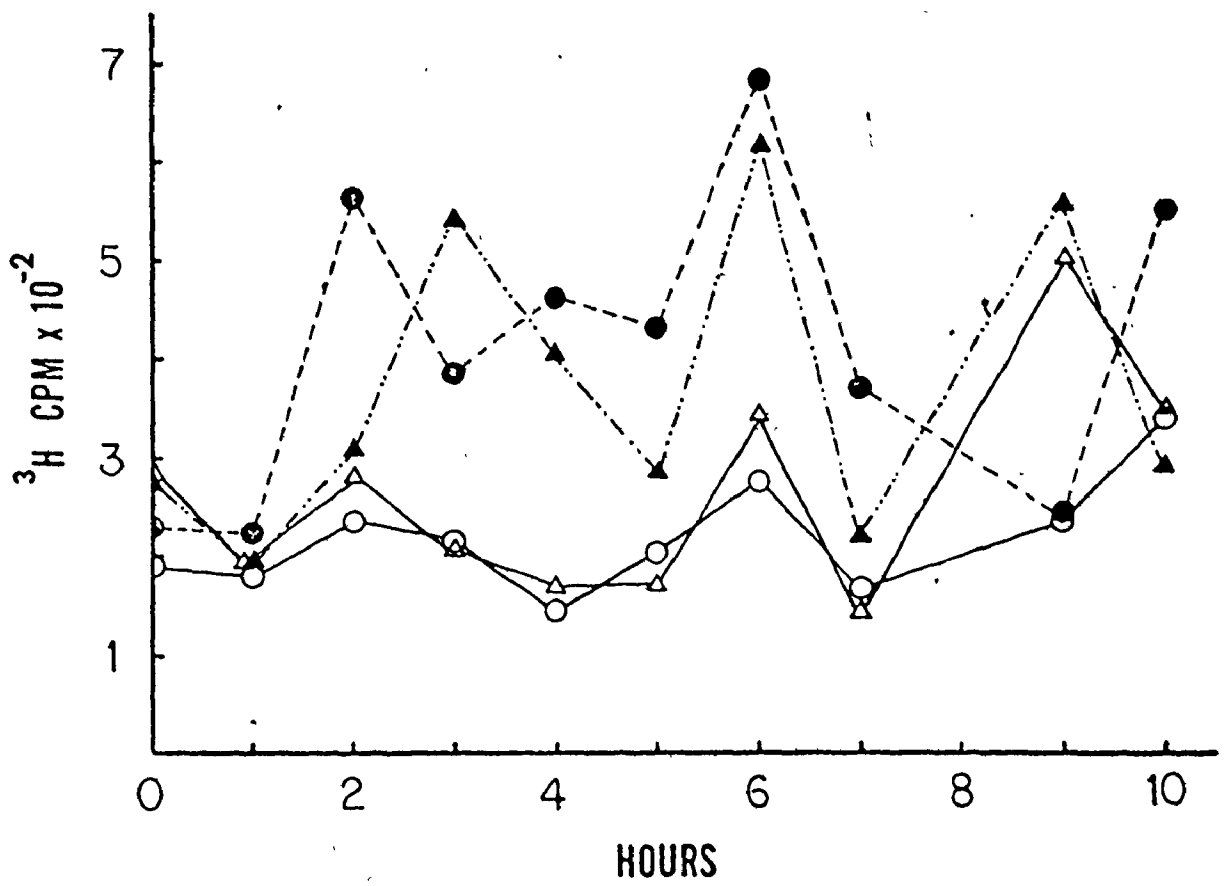


Figure 9

DNA synthesis in the nuclear fraction of vaccinia virus infected PE cells. Confluent monolayers of adherent rabbit PE cells (35 mm culture dish) were infected with 3.5×10^7 PFU of vaccinia virus for 30 minutes at 37°C. The virus was removed and 0.5 ml of MEM + 2% FCS was added to the plates which were incubated at 37°C. At hourly intervals, the cells were pulsed for 5 minutes with 5 μ c/ml of 3 H thymidine. The medium was removed and the cells were scraped into 1 ml of cold RSB and allowed to swell for 10 minutes in an ice bath. The cells were ruptured by 10 strokes of a Dounce homogenizer and the nuclear and cytoplasmic fractions separated by spinning at 800 x g for 5 minutes. The nuclear pellet was resuspended in a small amount of buffer, precipitated with 15% TCA and counted. Normal PE cells infected with vaccinia virus (●---●); normal PE cells mock infected (Δ ... Δ); activated PE cells infected with vaccinia virus (\blacktriangle — \blacktriangle); activated PE cells mock infected (○---○). These data represent the mean of two experiments. They are representative of the data obtained from two other experiments performed for 6 hours of incubation.

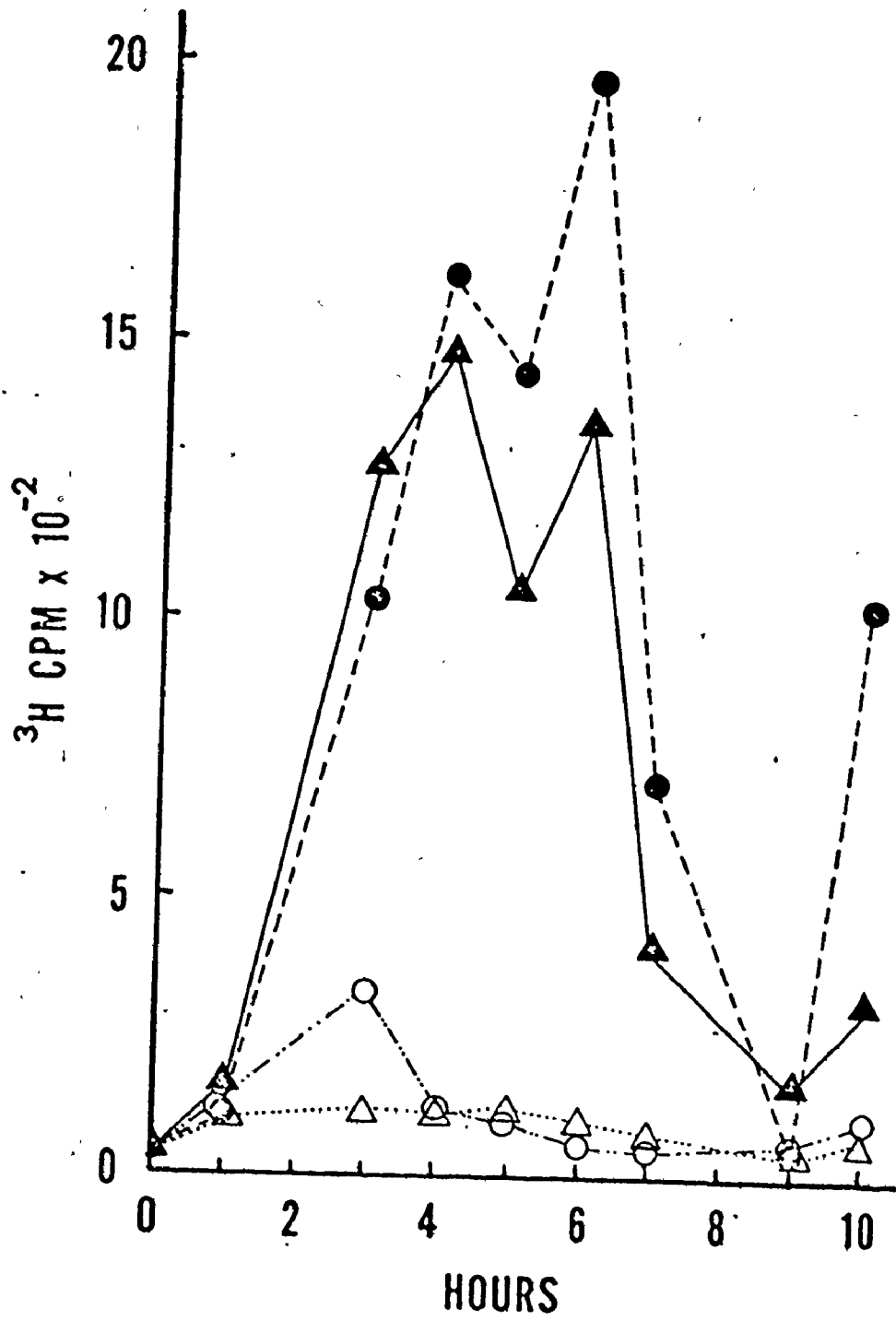
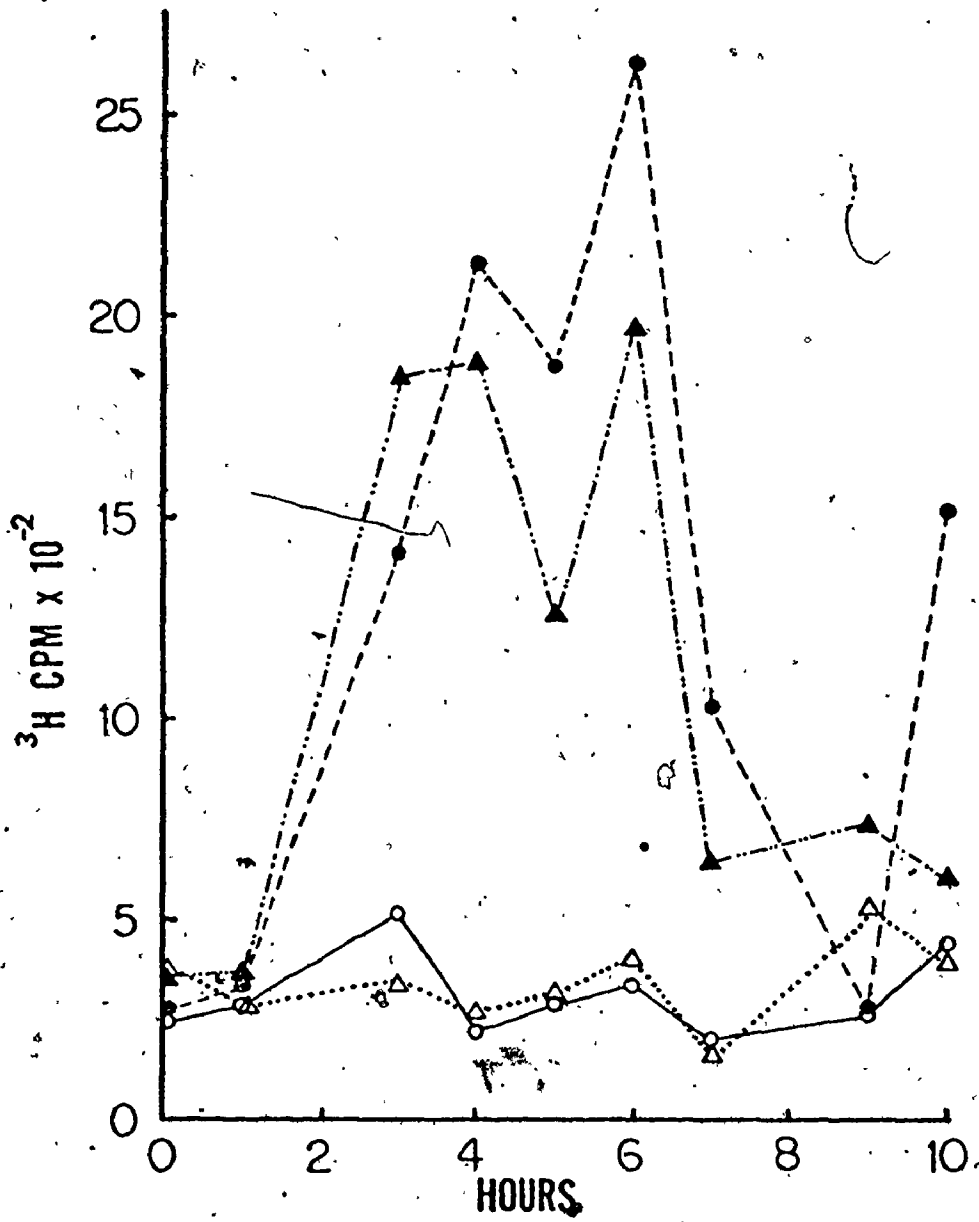


Figure 10

DNA synthesis in PE cells infected with vaccinia virus. Confluent monolayers of adherent rabbit PE cells (35 mm culture dish) were infected with 3.5×10^7 PFU of vaccinia virus for 30 minutes at 37°C. The virus was removed and 0.5 ml of MEM + 2% FCS was added to the plates which were incubated at 37°C. At hourly intervals, the cells were pulsed for 5 minutes with 5 μ c/ml of 3 H thymidine. The medium was removed and the cells were scraped into 1 ml of cold RSB and allowed to swell for 10 minutes in an ice bath. The cells were ruptured by 10 strokes of a Dounce homogenizer and the nuclear and cytoplasmic fractions separated by spinning at 800 x g for 5 minutes. The cytoplasmic and nuclear fractions were precipitated with 15% TCA and counted separately. The counts for the cytoplasmic and nuclear fractions for each time period were added together to give total cell counts. Normal PE cells infected with vaccinia virus (●---●); normal PE cells mock infected (○—○); activated PE cells infected with vaccinia virus (▲---▲); activated PE cells mock infected (△···△). These data represent the mean of two experiments. They are representative of the data obtained from two other experiments performed for 6 hours of incubation.



in activated macrophages. Therefore, the production of viral specific proteins in PE cells was evaluated. Initially, the cells were examined for antigens using indirect immunofluorescence. Both infected activated and infected normal macrophages were found to produce an identical pattern of fluorescence with a similar time course of appearance of antigen. The appearance of the virus specific antigen is given in Table 3. At 6 hours, both the activated and normal cells contained a few positive cells displaying dim, diffuse cytoplasmic fluorescence. By 9 hours, approximately 1 in 40 cells of both cell types displayed this diffuse cytoplasmic fluorescence (Figure 11a). At 22 hours, many more of the vaccinia virus infected normal macrophages than activated macrophages had detached from the coverslips; however, the fluorescing cells were identical in appearance and contained a bright cytoplasmic fluorescence which filled the cells. No vaccinia virus infected normal macrophages remained on the coverslip at 48 hours. The remaining infected activated macrophages at 48 hours were similar in appearance to those at 22 hours (Figure 11b). The positive cells were filled with a bright cytoplasmic fluorescence. At all time periods, infected cells incubated with normal rabbit serum did not stain (Figure 11c) and noninfected cells stained with the antiserum to vaccinia virus were negative.

Two dimensional immunodiffusion (Ouchterlony procedure) was performed on agar coated slides using plastic templates. In normal PE cells infected with vaccinia virus, a faint band could be detected in cells solubilized 4 hours after infection (Figure 12, well C) and by 6 hours, 4 bands could be readily detected (Figure 12, well D). Activated PE cells infected with vaccinia virus also produced virus antigens. Faint bands could again be initially detected at 4 hours after infection (Figure 13, well A). By 6 hours after infection, 4 bands were detected and additional bands were detectable 9 hours after infection (Figure 13, wells B and C). As is evident from Figure 13, well E, the antiserum contained antibodies to fetal calf serum. However, the 2 bands detected in the immunodiffusion plate did not identify with any of those present in the preparations of infected cells. Lysates of primary rabbit kidney

TABLE III

IMMUNOFLUORESCENCE IN PE CELLS INFECTED WITH VACCINIA VIRUS

Time After Infection (Hour)	Fluorescence Patterns	
	Activated Macrophage (Vaccinia Virus Infected)	Normal Macrophage (Vaccinia Virus Infected)
1/2	Negative	Negative
2	Negative	Negative
4	Negative	Negative
6	Dim, diffuse cytoplasmic fluorescence	Dim, diffuse cytoplasmic fluorescence
8	Dim cytoplasmic fluorescence	Dim cytoplasmic fluorescence
9	Dim cytoplasmic fluorescence	Dim cytoplasmic fluorescence
22	Bright cytoplasmic fluorescence	Bright cytoplasmic fluorescence
48	Bright cytoplasmic fluorescence	No cells remaining

Figure 11

Immunofluorescent staining of rabbit macrophages infected with vaccinia virus. Rabbit PE cells were infected with 2.5 PFU vaccinia virus per cell, allowed to adhere to coverslips and incubated at 37°C. The cells were stained with vaccinia virus immune rabbit serum or normal rabbit serum (NRS) and then with fluorescein conjugated goat antirabbit gamma globulin. a. normal macrophage infected with vaccinia virus, 9 hr incubation; b. activated macrophage infected with vaccinia virus, 48 hr incubation; c. activated macrophage infected with vaccinia virus and stained with NRS, 22 hr incubation.

FIG.11a



11b



FIG. 11c



Figure 12

Immunodiffusion reaction of normal PE macrophages infected with vaccinia virus. Adherent normal PE cells were infected with vaccinia virus, incubated at 37°C and lysed by freeze-thawing and sonication. Center well - vaccinia virus immune rabbit serum;
A. 0 hr incubation; B. 2 hr incubation; C. 4 hr incubation;
D. 6 hr incubation.

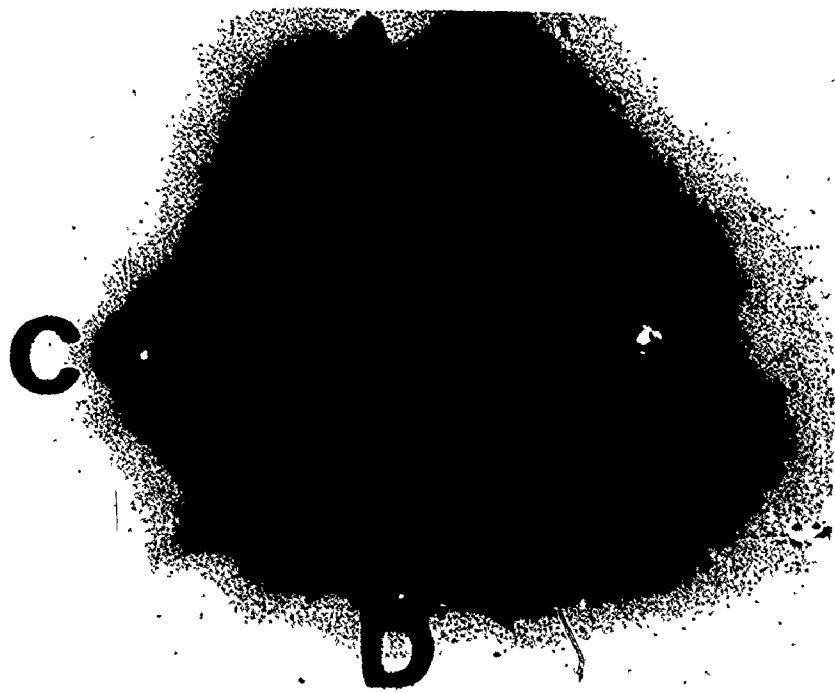
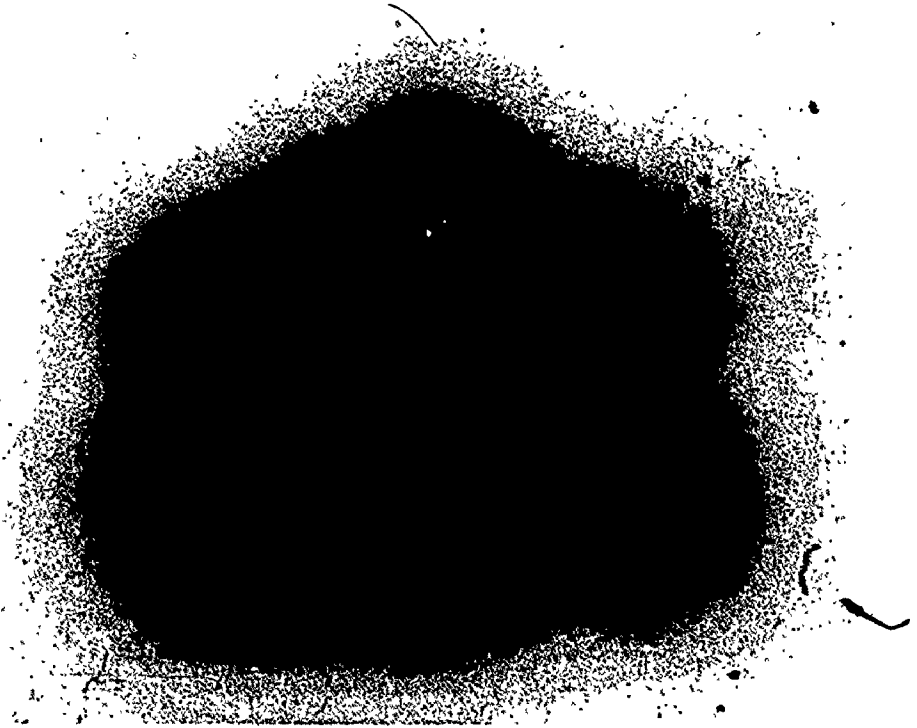


Figure 13

Immunodiffusion reaction of activated PE macrophages infected with vaccinia virus. Adherent activated PE cells or primary rabbit kidney cells (PRK) were infected with vaccinia virus, incubated at 37°C and lysed by freeze-thawing and sonication. Center well - vaccinia virus immune rabbit serum; A. activated macrophage, 4 hr incubation; B. activated macrophage, 6 hr incubation; C. and F. activated macrophages, 9 hr incubation; D. PRK, 18 hr incubation; E. fetal calf serum.



cells, which had been infected with vaccinia virus and incubated 18 hours, yielded a strong band which was identical to one of the bands produced 9 hours after infection of activated PE cells (Figure 13, well D and C).

A similar pattern of immunodiffusion bands was seen when lysates of infected Vero cells were used as antigens (Figure 14). As in the macrophage system, there were no detectable bands before 4 hours of infection. At 4 hours, there were three strong bands which increased in number at 6 and 8 hours after infection. Figure 14 also displays the immunodiffusion pattern of lysates of primary rabbit kidney cells 18 hours after infection with vaccinia virus.

To examine individual polypeptide synthesis, lysates of infected cells were run on 12.5% slab PAGE which were stained for protein and then prepared for fluorography. The protein staining patterns of vaccinia virus infected and noninfected cells were identical. The staining pattern of activated and normal macrophages also appeared identical. Therefore, vaccinia virus specific protein synthesis was detected by pulsing the cells for 30 minutes with ^3H amino acids at two hour intervals.

An autoradiograph of a SDS-PAGE gel of the cell lysates of virus infected activated and infected normal macrophages is shown in Figure 15. The infected cells were pulsed with ^3H amino acids for 30 minutes at 0, 2, 4, 6, and 7 hours after infection. During the first 30 minutes of incubation, very little protein synthesis took place (lanes A and F). At 2 hours after infection, a few new identically migrating polypeptides were formed in both the activated and normal macrophages (lanes B and G). By 4 hours, protein synthesis in the vaccinia virus infected macrophages was proceeding at a rapid rate in both activated and normal macrophages (lane C and H). Many new polypeptides continued to be synthesized at 6 and 7 hours after infection (lanes D, E, I, and J). The gel profiles of polypeptides synthesized in vaccinia virus infected activated and normal macrophages were remarkably similar. However, several differences between the activated and normal virus infected

Figure 14

Immunodiffusion reaction of Vero cells infected with vaccinia virus. Monolayers of Vero cells or primary rabbit kidney cells (PRK) were infected with vaccinia virus, incubated at 37°C and lysed by freeze-thawing and sonication. Center well - vaccinia virus immune rabbit serum. A. Vero cell, 0 hr incubation; B. Vero cell, 2 hr incubation; C. Vero cell, 4 hr incubation; D. Vero cell, 6 hr incubation; E. Vero cell, 8 hr incubation; F. PRK, 18 hr incubation.

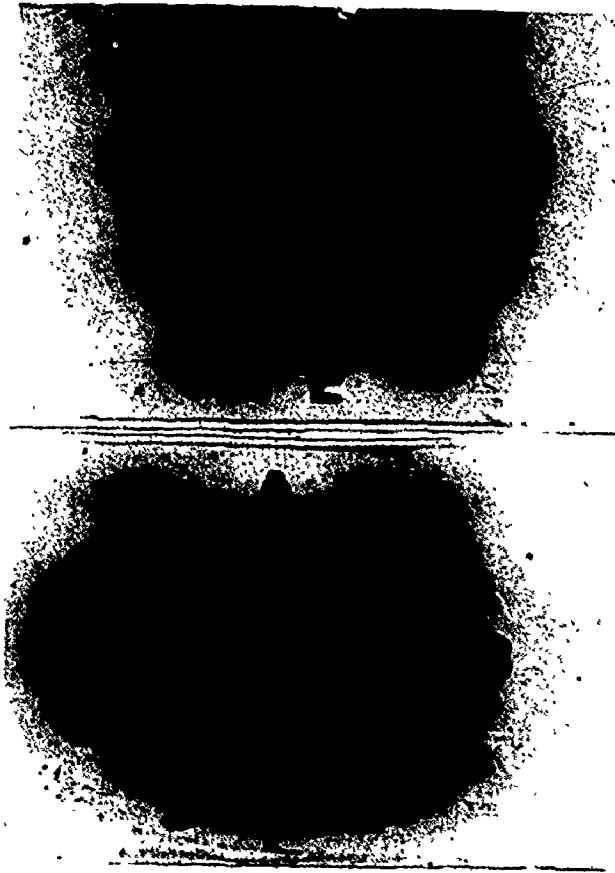


Figure 15

The sequential appearance of polypeptides in rabbit macrophages infected with vaccinia virus. Adherent PE cells infected with vaccinia virus were pulsed for 30 minutes at various times after infection with 25 μ c/ml of 3 H amino acids in amino acid free MEM. After pulsing, the medium was removed, the cells were solubilized in slab gel sample preparation buffer and 100 μ l of sample was run on a 12.5% polyacrylamide gel containing 0.1% SDS. Radio-labelled polypeptides were detected by fluorography. A. normal macrophage, 0 hr incubation; B. normal macrophage, 2 hr incubation; C. normal macrophage, 4 hr incubation; D. normal macrophage, 6 hr incubation; E. normal macrophage, 7 hr incubation; F. activated macrophage, 0 hr incubation; G. activated macrophage, 2 hr incubation; H. activated macrophage, 4 hr incubation; I. activated macrophage, 6 hr incubation; J. activated macrophage, 7 hr incubation.

A B C D E F G H I J



— 2.5 cm

— 3.0 cm

macrophages were noted. These differences were seen best at 6 and 7 hours after infection. There was a doublet in the activated macrophages approximately 2.5 cm from the top of the gel while in the normal macrophage, only one band appeared. This is in the region of 65,000 daltons molecular weight as determined by running known standards on a similar gel. Another band at 3 cm from the top of the gel (\sim 60,000 daltons) is apparent in the activated macrophages but missing in the normal macrophages infected with virus.

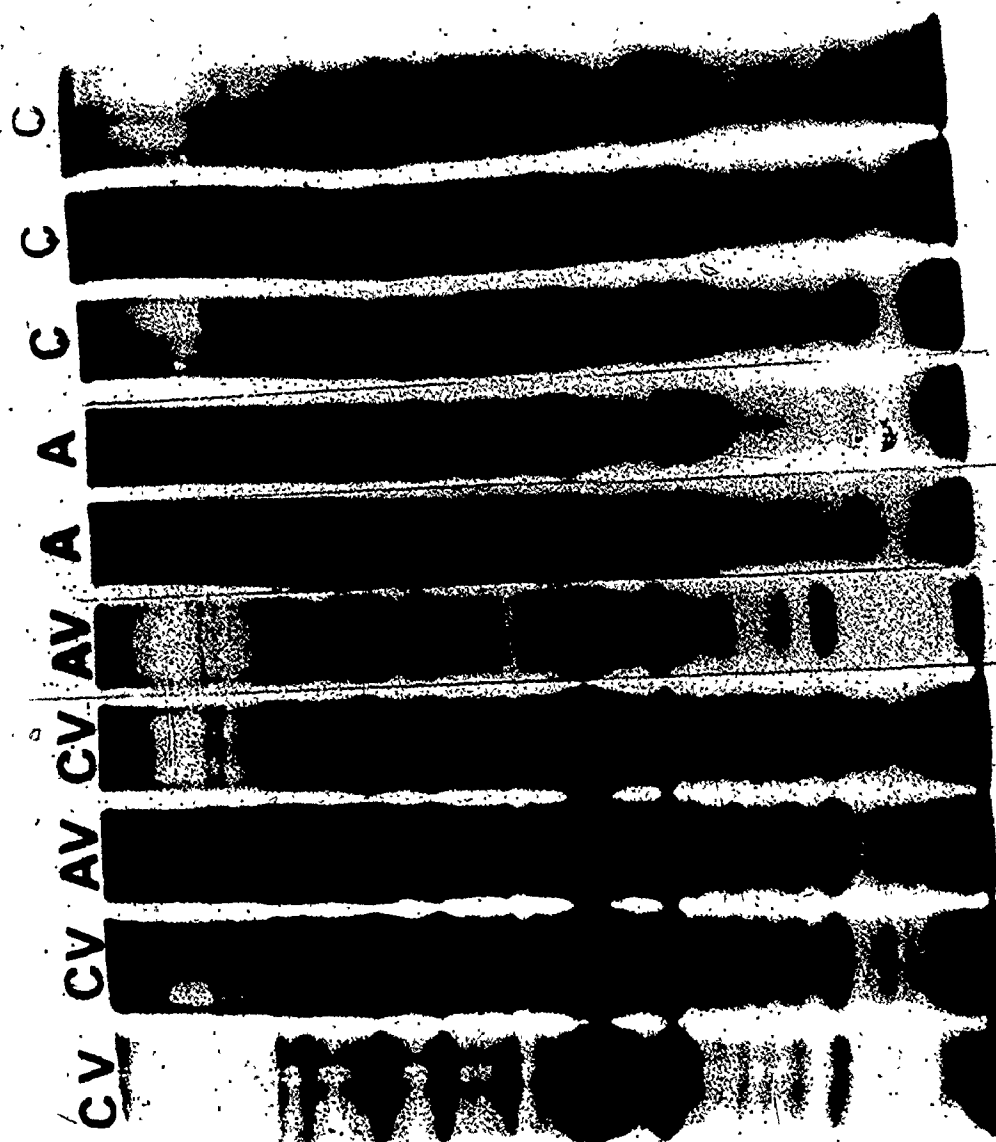
Gel profiles of cells from three other control rabbits and two other activated rabbits are shown in Figure 16. The cells were pulsed for 30 minutes at 6 hours after incubation with vaccinia virus or mock infected. Several polypeptide bands were present in the infected activated macrophages (AV) as compared to the infected control macrophages (CV). These bands were located at 2.3 cm and 3.5 cm from the top of the gel and corresponded to the bands described in Figure 15. Also seen is a band in the infected activated macrophages at 7.7 cm which is missing from the infected control macrophages and the absence of a band at 8.0 cm which is present in the infected control macrophages. The gel profiles of polypeptides synthesized in noninfected activated (A) and control (C) macrophages 6 hours after incubation revealed no differences in their protein synthesis.

The autoradiograph of a SDS-PAGE gel of infected Vero cells is shown in Figure 17. More polypeptides were produced in the Vero cells than in the macrophages at all times after infection. The majority of new polypeptides in Vero cells appeared at 4 hours after infection. The 6 hour profile was very similar to 4 hours while at 8 hours the rate of polypeptide synthesis had declined.

Posttranslational cleavage has been reported for several vaccinia virus proteins. In order to study protein cleavage in macrophages, pulse-chase experiments were performed. Cells infected with vaccinia virus were pulsed with ^3H amino acids for 30 minutes at 6 hours after infection and then chased for 2, 4, or 6 hours with unlabelled amino acids. Slab SDS-PAGE were then run on the cell lysates and autoradio-

Figure 16

Polypeptide synthesis in rabbit macrophages 6 hours after infection with vaccinia virus. Adherent PE cells from three control rabbits and two activated rabbits were infected with vaccinia virus or mock infected and incubated at 37°C for 6 hours. At 6 hours, they were pulsed for 30 minutes with 25 µc/ml of ³H amino acids mixture in amino acid free MEM. After pulsing, the medium was removed and the cells were solubilized in slab gel sample preparation buffer. One hundred lambda of sample was run on a 12.5% polyacrylamide gel containing 0.1% SDS. Radiolabelled polypeptides were detected by fluorography. CV. control macrophage, vaccinia virus infected; AV. activated macrophage, vaccinia virus infected; C. control macrophage, mock infected; A. activated macrophage, mock infected.



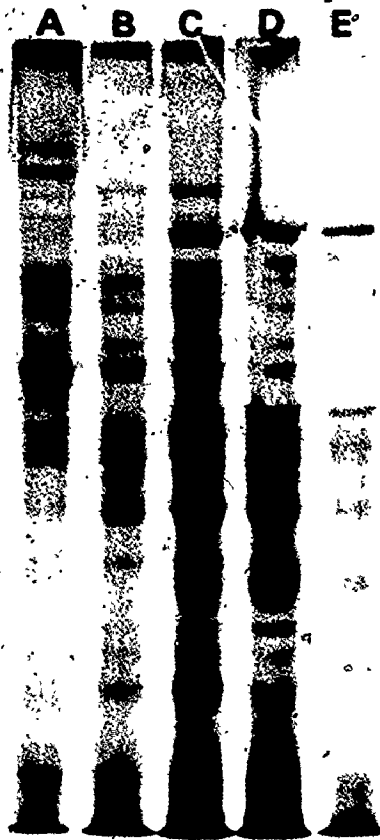
2.3 cm —

3.5 cm —

7.7 cm =
8.0 cm =

Figure 17

The sequential appearance of polypeptides in Vero cells infected with vaccinia virus. Vero cells infected with vaccinia virus were pulsed for 30 minutes at various times with 25 $\mu\text{C}/\text{ml}$ of ^3H amino acids in amino acid free MEM. After pulsing, the medium was removed, the cells were solubilized in slab gel sample preparation buffer and 100 μl . of sample was run on a 12.5% polyacrylamide gel containing 0.1% SDS. Radiolabelled polypeptides were detected by fluorography. A. 0 hr incubation; B. 2 hr incubation; C. 4 hr incubation; D. 6 hr incubation; E. 8 hr incubation.



graphs made. Figure 18 shows the gel of Vero cells infected with vaccinia virus. Lane A represents the 30 minute pulse at 6 hours after infection with no chase while lanes B, C, and D are chases of 2, 4, and 6 hours respectively. Two differences were noted in the gel profiles after the 6 hour chase. In the second group of polypeptides from the top of the gel (1.6 cm), there appeared to be a decrease in the lower band and the appearance of a new band slightly above it. This was first apparent after 4 hours of chase and was strengthened at 6 hours. The second difference noted was the appearance of a band at 7.8 cm from the top of the gel after the 6 hour chase. This band was not present at earlier times.

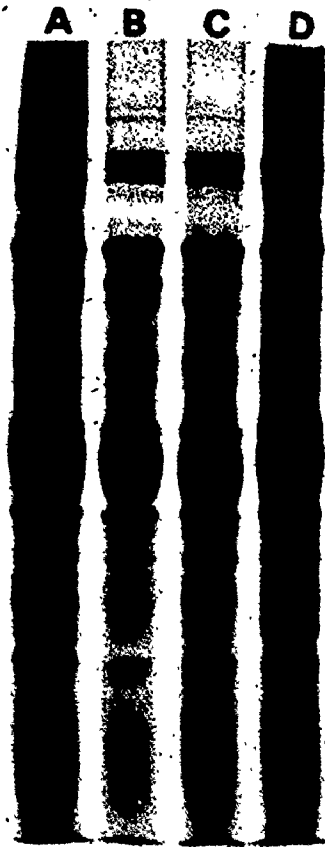
The results of an identical experiment using normal and activated macrophages infected with virus is shown in Figure 19. The activated macrophage lysates (lanes E-H) exhibited only a small change in protein bands during the 6 hour chase. The prominent band at 6.5 cm from the top of the gel after no chase (lane E) appeared to move slightly downward during the 6 hour chase. This was most apparent after the full 6 hours of chase (lane H). A shift in polypeptide band position was also seen in the infected normal macrophages during the 6 hour chase. The band at 6.5 cm from the top of the gel decreased in intensity during the 6 hour chase while another band slightly above it appeared starting at 4 hours of chase (lane C). The new band became one of the major bands in the infected normal macrophages after the 6 hour chase (lane D).

VI. Detection of Virus Particles

Since viral DNA and proteins were synthesized in activated macrophages, an attempt was made to detect the production of virus particles. Vaccinia virus can be readily purified. The virus has a characteristic sedimentation profile in a 25% to 40% continuous sucrose gradient (Figure 20). Therefore, vaccinia virus was replicated in the presence of ^{14}C amino acids or ^3H thymidine and the harvests were tested on sucrose gradients for peaks of radioactivity corresponding with that of the virus. At 8 hours after infection, infected Vero cells displayed

Figure 18

Posttranslational changes of polypeptides synthesized in Vero cells infected with vaccinia virus. Vero cells infected with vaccinia virus were pulsed with 25 $\mu\text{C}/\text{ml}$ of ^3H amino acids for 30 minutes at 6 hours after infection. The radioactive medium was then removed, MEM + 10% FCS added and the cells incubated at 37°C for their various chase periods. After the chase period, the cells were solubilized in slab gel sample preparation buffer and 100 μl of sample was run on a 12.5% polyacrylamide gel containing a 0.1% SDS. Radiolabelled polypeptides were detected by fluorography. A. 0 hr chase period; B. 2 hr chase period; C. 4 hr chase period; D. 6 hr chase period.

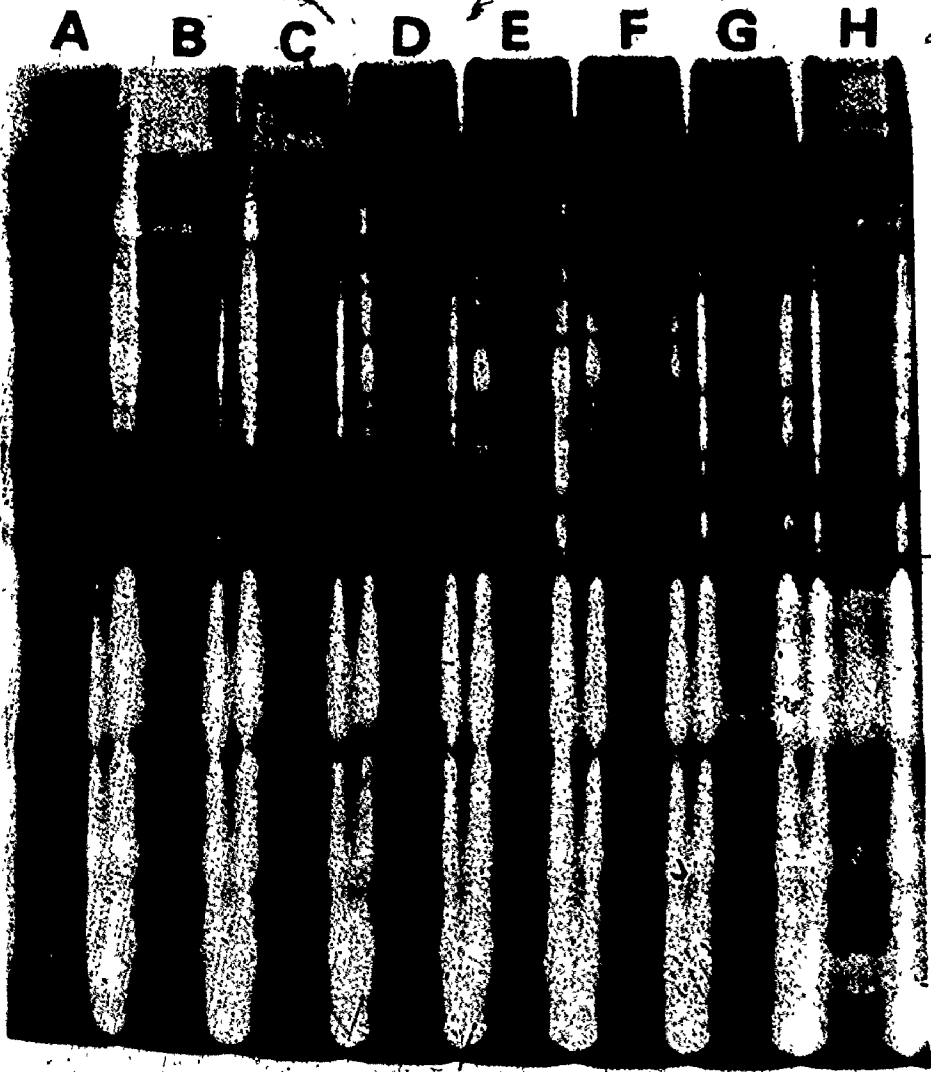


—1.6 cm

—7.8 cm

Figure 19

Posttranslational changes of polypeptides synthesized in rabbit macrophages infected with vaccinia virus. Adherent PE cells were pulsed for 30 minutes with 25 $\mu\text{C}/\text{ml}$ of ^3H amino acids at 6 hours after infection with vaccinia virus. The radioactive medium was removed, MEM + 10% FCS was added and the cells further incubated at 37°C for their various chase periods. After the chase period, the cells were solubilized with slab gel sample preparation buffer and 100 μl of sample was run on a 12.5% polyacrylamide gel containing 0.1% SDS. Radiolabelled polypeptides were detected by fluorography. A. normal macrophage, 0 hr chase period; B. normal macrophage, 2 hr chase period; C. normal macrophage, 4 hr chase period; D. normal macrophage, 6 hr chase period; E. activated macrophage, 0 hr chase period; F. activated macrophage, 2 hr chase period; G. activated macrophage, 4 hr chase period; H. activated macrophage, 6 hr chase period.



A

B

C

D

E

F

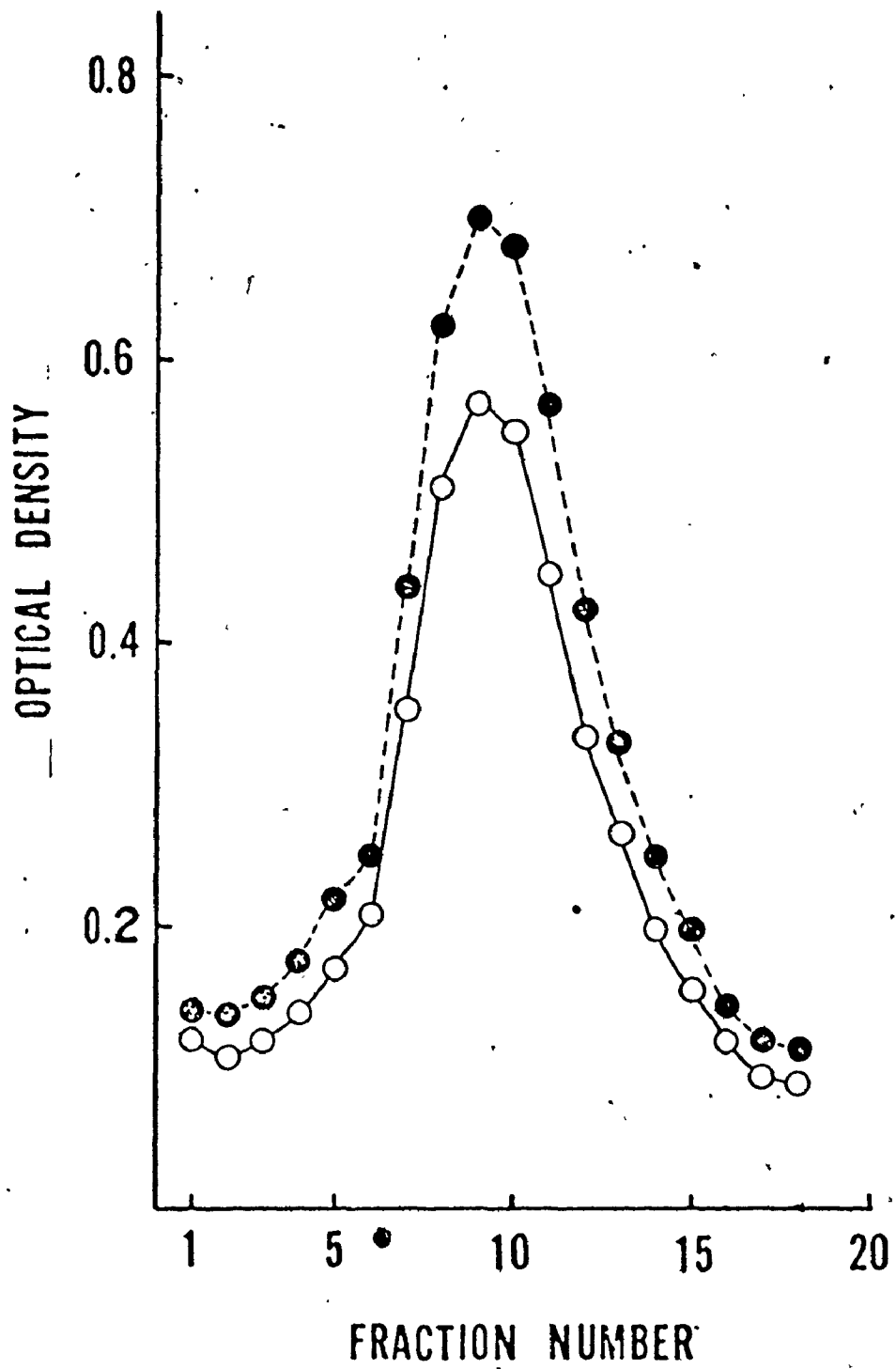
G

H

6.5 cm

Figure 20

Sedimentation profile of purified vaccinia virus in a 25 to 40% continuous sucrose gradient. Purified vaccinia virus was sedimented in a 25 to 40% continuous sucrose gradient for one hour of $14,000 \times g$. Forty drop fractions were collected by bottom puncture and the optical densities at 260 nm and 280 nm were recorded. 260 nm (●---●), 280 nm (○—○).



a rise in ^{14}C counts toward the top of the gradient representing small aggregates of protein (Figure 21). At 22 hours, there was a larger accumulation of putative virus particles as indicated by a distinct plateau of protein in the region of the virus markers. The normal macrophages infected with vaccinia virus displayed only a minimal formation of particles at 8 hours (Figure 22). At 22 hours, however, there was a definite peak of vaccinia virus sized particles. The infected activated macrophages gave a decidedly different type of profile as shown in Figure 23. There was no peak of activity at any place in the gradient but only a steady rise in the labelled amino acids from the bottom to the top of the gradient. The 8 hour and 22 hour curves had similar slopes with the 22 hour incubation time profile exhibiting a larger accumulation of labelled protein throughout the gradient.

(Vaccinia virus infected cells were continuously labelled for 8 or 22 hours with ^3H thymidine and the contents placed on a 25 to 40% sucrose gradient in order to detect the formation of DNA containing particles. To assure that the particles were vaccinia virus specific, each gradient fraction was tested for complement fixing (CF) activity using specific antiserum. By 8 hours, infected Vero cells produced appreciable amounts of labelled DNA with sedimentation characteristics similar to vaccinia virus (Figure 24). This was confirmed by the CF test which demonstrated a peak of virus antigen in the middle of the same gradient. By 22 hours after the infection of Vero cells, the amount of DNA containing particles had sharply declined. There was also a loss of detectable virus antigen in the gradient. Figure 25 shows the results of vaccinia virus infection in normal macrophages. At 8 hours after infection, there were no DNA containing particles nor detectable vaccinia virus antigen in the gradient. However, a large peak of DNA containing particles was observed in the gradient containing preparations harvested at 22 hours. Vaccinia virus antigen was also detected beginning in the fractions where the DNA peak rose and continued to the top of the gradient. No real peak of DNA containing particles were produced by activated macrophages infected with vaccinia virus (Figure 26).

Figure 21

Amino acid labelled virus particles detected in Vero cells infected with vaccinia virus. Vero cells infected with vaccinia virus were continuously labelled with 1 $\mu\text{C}/\text{ml}$ of ^{14}C amino acids for 8 or 22 hours. The cells were then lysed by freeze-thawing, cell debris removed by low speed centrifugation and the cell lysate diluted in 20 ml of 0.01 M Tris, pH 9. The diluted supernatant was spun at 25,000 x g for one hour and the pellet resuspended in a small amount of buffer. This was layered on a continuous 25 to 40% sucrose gradient along with 25 lambda of purified vaccinia virus labelled with ^3H thymidine and spun at 14,000 x g for one hour. Twenty five drop fractions were collected by bottom puncture, solubilized with BBS-3 and the whole fraction counted. ^{14}C amino acids, 8 hrs of infection ($\bullet\text{---}\bullet$); ^{14}C amino acids, 22 hrs of infection ($\text{O}\text{---}\text{O}$); ^3H thymidine labelled purified vaccinia virus ($\Delta\text{---}\Delta$). These data are representative of duplicate experiments.

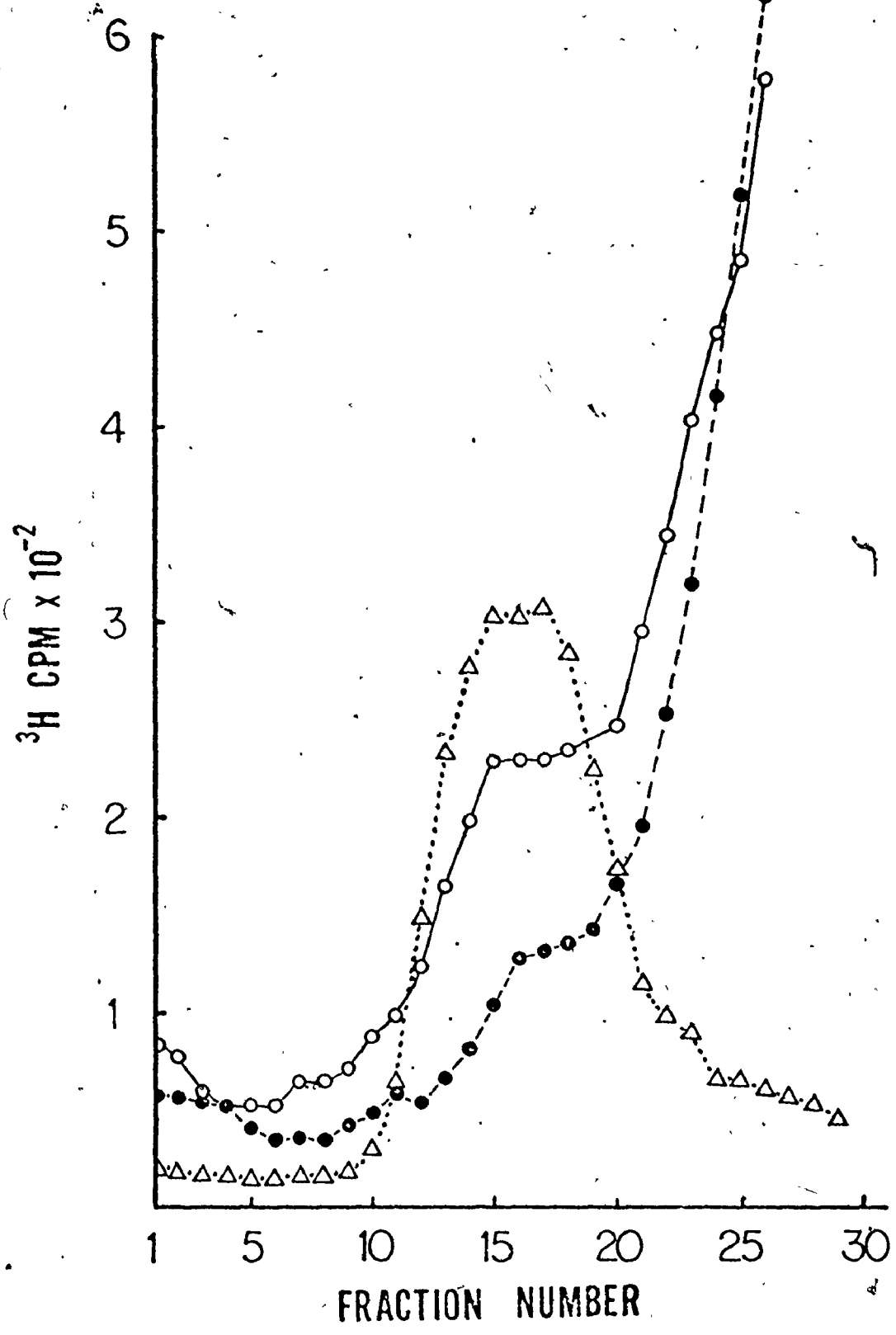


Figure 22.

Amino acid labelled virus particles detected in normal macrophages infected with vaccinia virus. Adherent normal PE cells infected with vaccinia virus were continuously labelled with 1 $\mu\text{C}/\text{ml}$ of ^{14}C amino acids for 8 or 22 hours. The cells were then lysed by freeze-thawing, cell debris removed by low speed centrifugation and the cell lysate diluted in 20 ml of 0.01 M Tris, pH 9. The diluted supernatant was spun at 25,000 \times g for one hour and the pellet resuspended in a small amount of buffer. This was layered on a continuous 25 to 40% sucrose gradient along with 25 lambda of purified vaccinia virus labelled with ^3H thymidine and spun at 14,000 \times g for one hour. Twenty five drop fractions were collected by bottom puncture, solubilized with BBS-3 and the whole fraction counted. ^{14}C amino acids, 8 hrs of infection ($\bullet\text{---}\bullet$); ^{14}C amino acids, 22^{hrs} of infection ($\square\cdots\square$); ^3H thymidine labelled purified vaccinia virus ($\triangle\text{---}\triangle$). These data are representative of duplicate experiments.

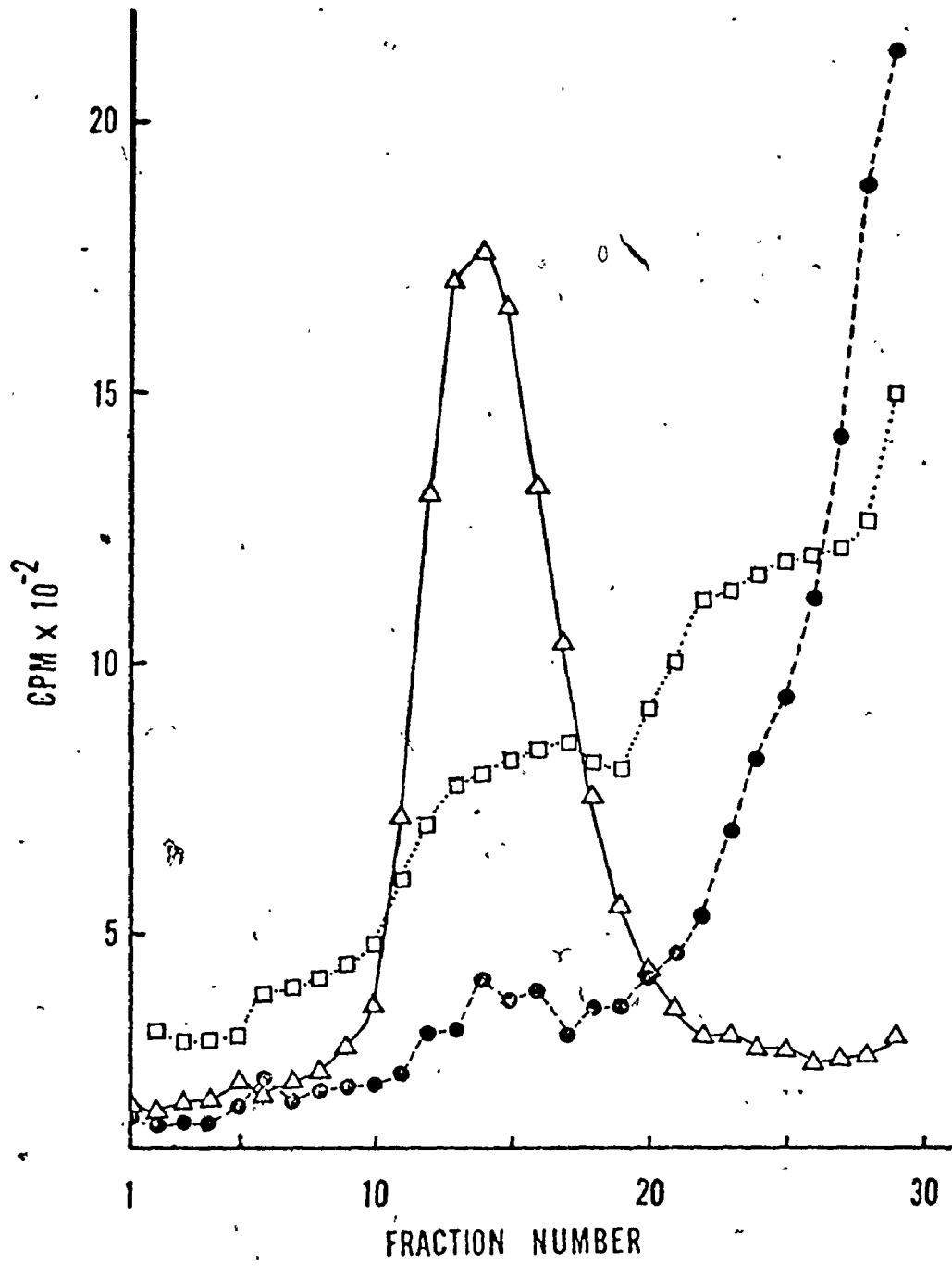


Figure 23

Amino acid labelled virus particles detected in activated macrophages infected with vaccinia virus. Adherent activated PE cells infected with vaccinia virus were continuously labelled with 1 μ c/ml of 14 C amino acids for 8 or 22 hours. The cells were then lysed by freeze-thawing, cell debris removed by low speed centrifugation and the cell lysate diluted in 20 ml of 0.01 M Tris, pH 9. The diluted supernatant was spun at 25,000 x g for one hour and the pellet resuspended in a small amount of buffer. This was layered on a continuous 25 to 40% sucrose gradient along with 25 lambda of purified vaccinia virus labelled with 3 H thymidine and spun at 14,000 x g for one hour. Twenty five drop fractions were collected by bottom puncture, solubilized with BBS-3 and the whole fraction counted. 14 C amino acids, 8 hrs of infection (●---●); 14 C amino acids, 22 hrs of infection (□---□); 3 H thymidine labelled purified vaccinia virus (△---△). These data are representative of duplicate experiments.

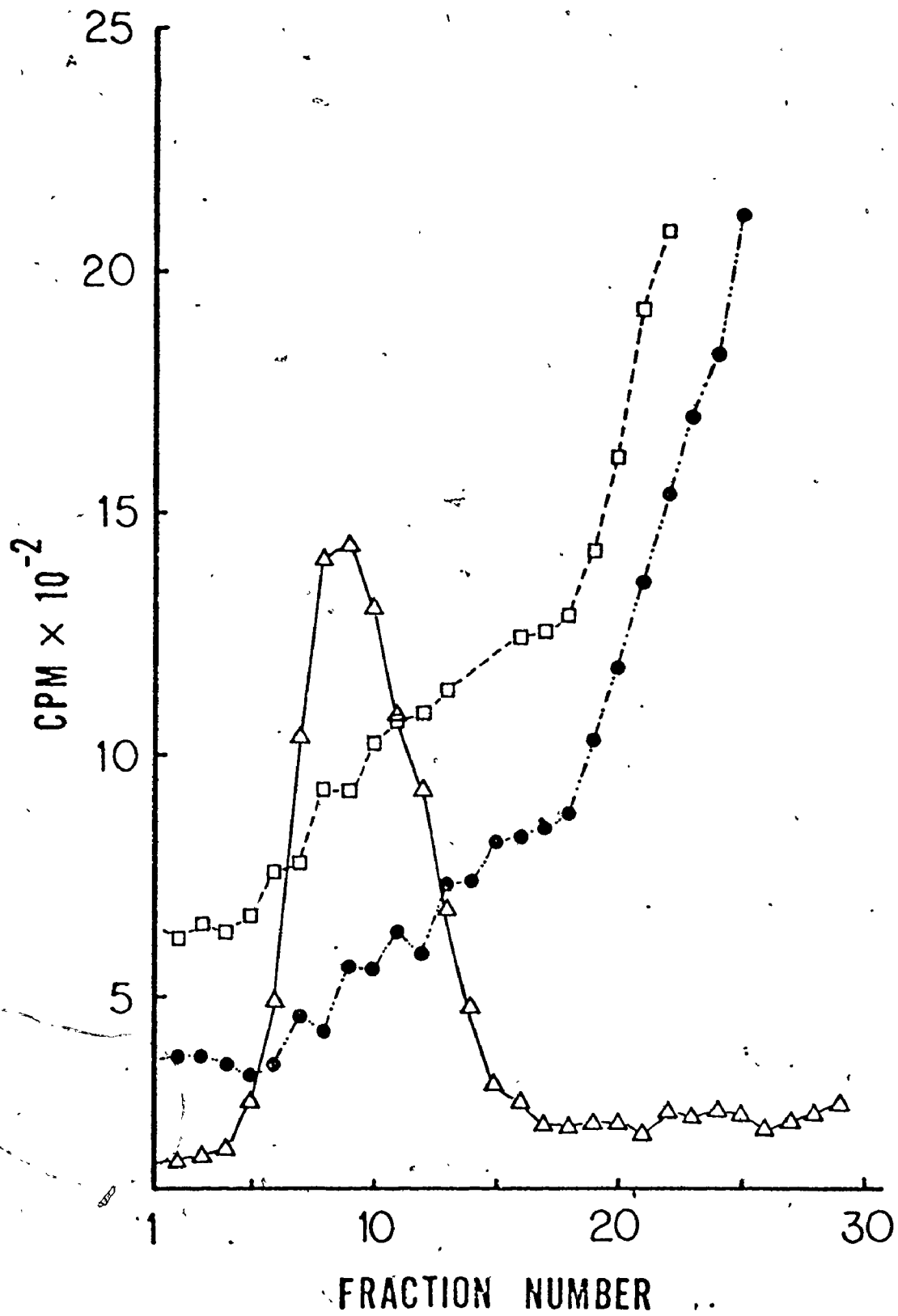


Figure 24

Thymidine labelled virus particles detected in Vero cells infected with vaccinia virus. Vero cells infected with vaccinia^v virus were continuously labelled with 5 μ c/ml of ³H thymidine for 8 or 22 hours. The cells were then lysed by freeze-thawing, cell debris removed by low speed centrifugation and the cell lysate diluted in 20 ml of 0.01 M Tris, pH 9. The diluted supernatant was spun at 25,000 x g for one hour and the pellet resuspended in a small amount of buffer. This was layered on a continuous 25 to 40% sucrose gradient and spun at 14,000 x g for one hour. Twenty five drop fractions were collected by bottom puncture. Fifty lambda of each fraction was removed and tested for vaccinia virus antigen using the complement fixation (CF) test. The remaining portion of each fraction was solubilized and counted for radioactivity. ³H thymidine, 8 hrs (●—●); ³H thymidine, 22 hrs (○---○); vaccinia virus antigen, 8 hrs (▲—▲); vaccinia virus antigen, 22 hrs (△---△). These data are representative of duplicate experiments.

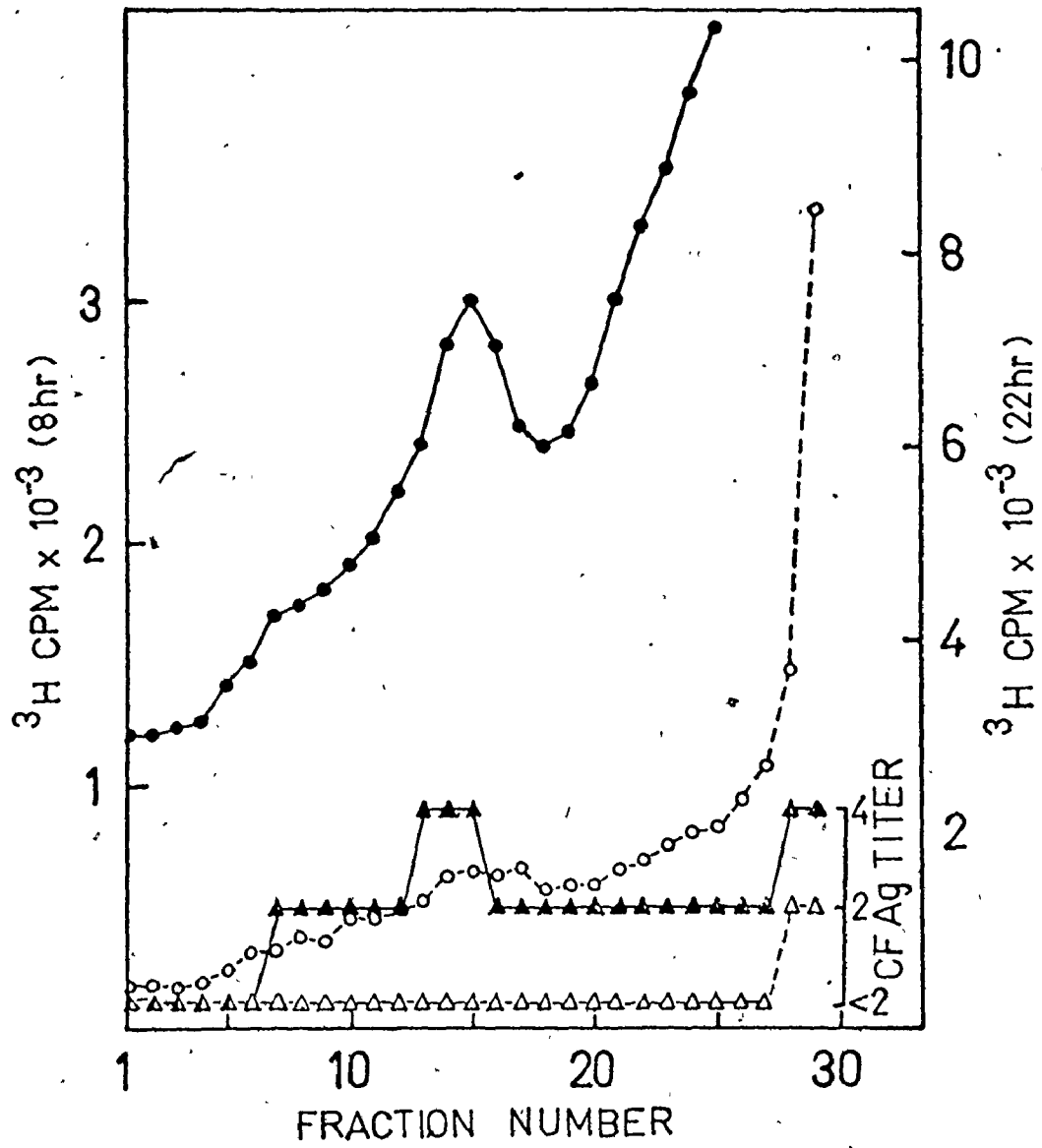


Figure 25

Thymidine labelled virus particles detected in normal macrophages infected with vaccinia virus. Adherent normal PE cells infected with vaccinia virus were continuously labelled with 5 μ C/ml of 3 H thymidine for 8 or 22 hours. The cells were then lysed by freeze-thawing, cell debris removed by low speed centrifugation and the cell lysate diluted in 20 ml of 0.01 M Tris, pH 9. The diluted supernatant was spun at 25,000 x g for one hour and the pellet resuspended in a small amount of buffer. This was layered on a continuous 25 to 40% sucrose gradient and spun at 14,000 x g for one hour. Twenty five drop fractions were collected by bottom puncture. Fifty lambda of each fraction was removed and tested for vaccinia virus antigen using the complement fixation (CF) test. The remaining portion of each fraction was solubilized and counted for radioactivity. 3 H thymidine, 8 hrs (●—●); 3 H thymidine, 22 hrs (○---○); vaccinia virus antigen, 8 hrs (▲—▲); vaccinia virus antigen, 22 hrs (△---△). These data are representative of duplicate experiments.

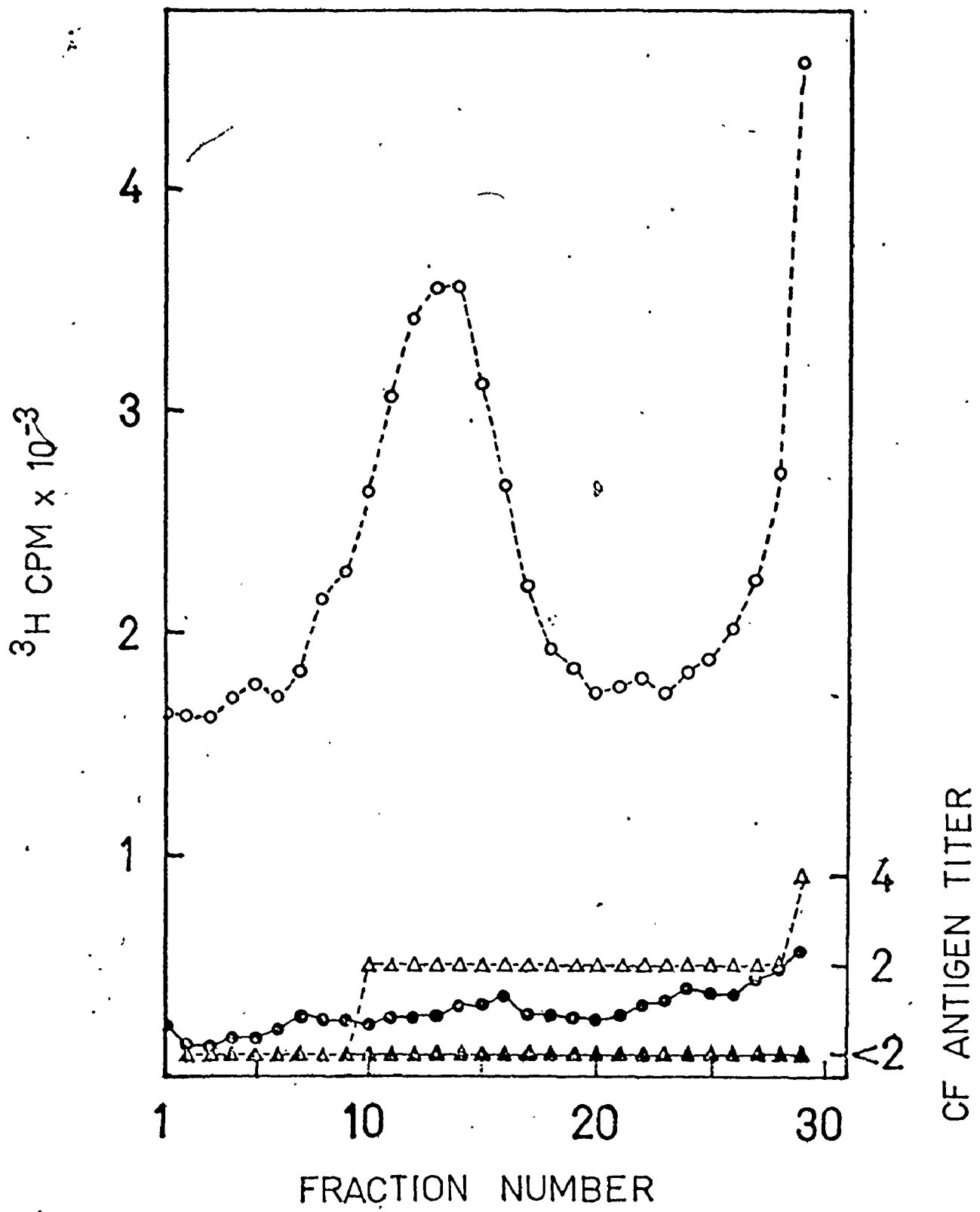
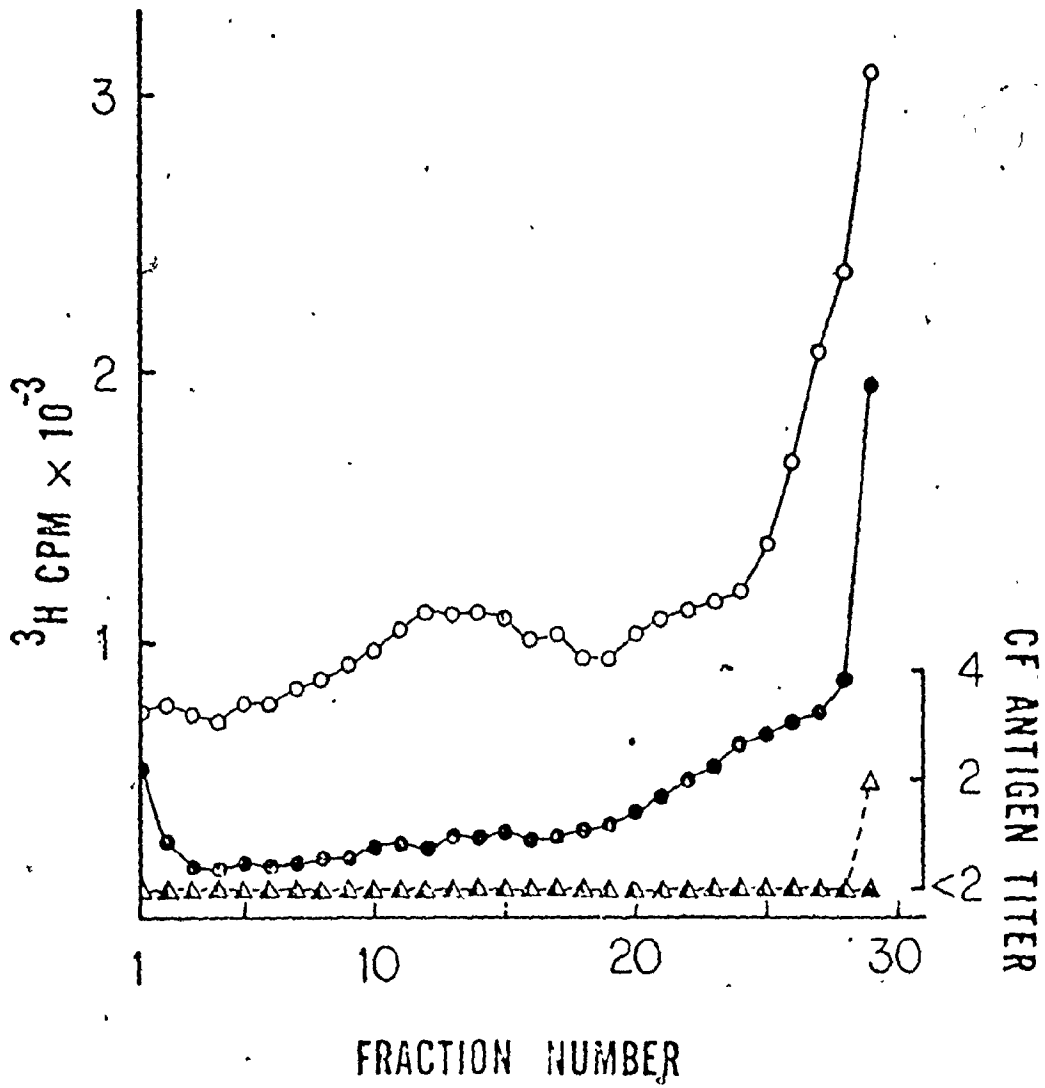


Figure 26

Thymidine labelled virus particles detected in activated macrophages infected with vaccinia virus. Adherent activated PE cells infected with vaccinia virus were continuously labelled with 5 μ c/ml of 3 H thymidine for 8 or 22 hours. The cells were then lysed by freeze-thawing, cell debris removed by low speed centrifugation and the cell lysate diluted in 20 ml of 0.01 M Tris, pH 9. The diluted supernatant was spun at 25,000 x g for one hour and the pellet resuspended in a small amount of buffer. This was layered on a continuous 25 to 40% sucrose gradient and spun at 14,000 x g for one hour. Twenty five drop fractions were collected by bottom puncture. Fifty lambda of each fraction was removed and tested for vaccinia virus antigen using the complement fixation (CF) test. The remaining portion of each fraction was solubilized and counted for radioactivity. 3 H thymidine, 8 hrs (●—●); 3 H thymidine, 22 hrs (○---○); vaccinia virus antigen, 8 hrs (▲—▲); vaccinia virus antigen, 22 hrs (△---△). These data are representative of duplicate experiments.



The results presented above suggest that either vaccinia virus particles are not produced in activated macrophages or that the particles produced are destroyed by lysosomal enzymes. Therefore, vaccinia virus was incubated with 10% suspensions of PE cell lysates and assayed periodically for loss of infectivity. The results, expressed as the percent reduction of titer when compared to the initial titer, are shown in Figure 27. There was a drop in the titer of vaccinia virus when it was incubated with the lysates of both activated and normal PE cells. Although there was a large variation from experiment to experiment, there appears to be no real difference between the effect of cell lysates from activated and normal PE cells on the infectivity of vaccinia virus. The reduction in virus titer was related to the length of time the virus was incubated with the cell lysates. Normal PE cell lysate reduced the titer by an average of 30% by 3 hours and 60% by 6 hours. Activated PE cell lysate caused an average of 28% reduction in vaccinia virus titer after 3 hours and a 46% reduction by 6 hours. The reduction in titers were greater when the virus was incubated with cell lysates than when incubated in balanced salt solution (Figure 27).

VII. Interferon Studies

The abortive infection of vaccinia virus in activated macrophages does not seem to be mediated by interferon since virus DNA and protein synthesis usually does not occur in interferon treated cells. However, few studies have dealt with interferon treated macrophages. Therefore, interferon was added to PE cells from normal rabbits and the replication of vaccinia virus in these cells was monitored. Two forms of interferon were used. One preparation was prepared in cultures of rabbit cells. This interferon had a 50% reduction titer of 1:10,000 and 1:1,000 against VSV and vaccinia virus, respectively (Figure 28). The other interferon preparation represented a serum sample collected from a rabbit 5 hours after intravenous injection of Poly (I)-Poly (C). This serum interferon had 50% reduction titers of 1:5000 and 1:100 against VSV and vaccinia virus, respectively.

Peritoneal exudate cells from normal rabbits were incubated

Figure 27

Action of 10% PE cell lysates on vaccinia virus. A 20% suspension by volume of freshly collected and washed PE cells was made in HBSS. The cells were lysed by freeze-thawing and the lysates clarified by low speed centrifugation. Equal volumes of vaccinia virus and cell lysate or HBSS were mixed to give a final cell lysate concentration of 10% and the mixture was divided into small tubes. The virus lysate mixtures were incubated at 37°C for various times until duplicate tubes were freeze-thawed and the amount of infectious virus assayed on Vero cells. Experiment 1 (▲—▲); Experiment 2 (●—●); Experiment 3 (△—△); Experiment 4 (○—○).

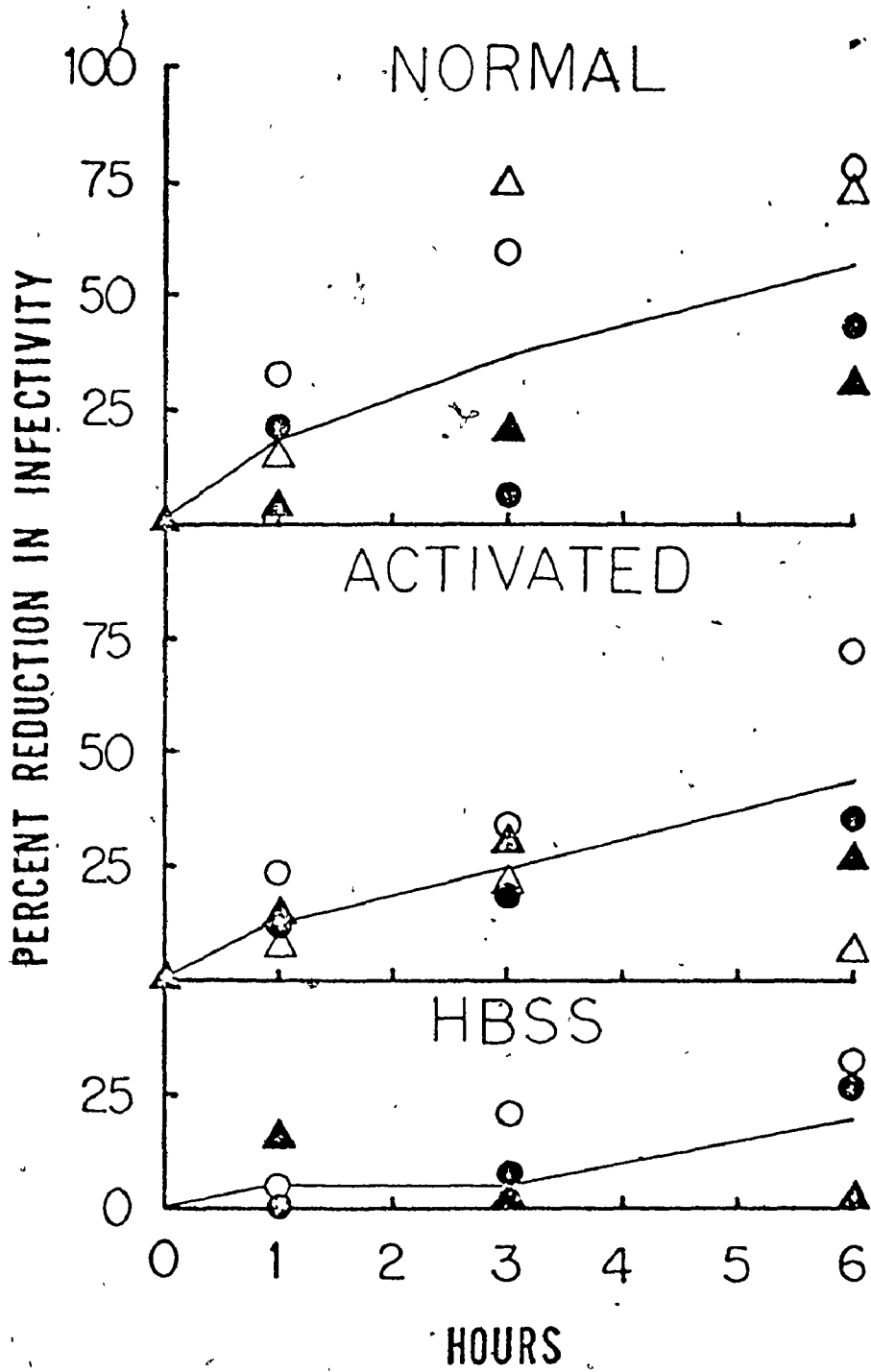
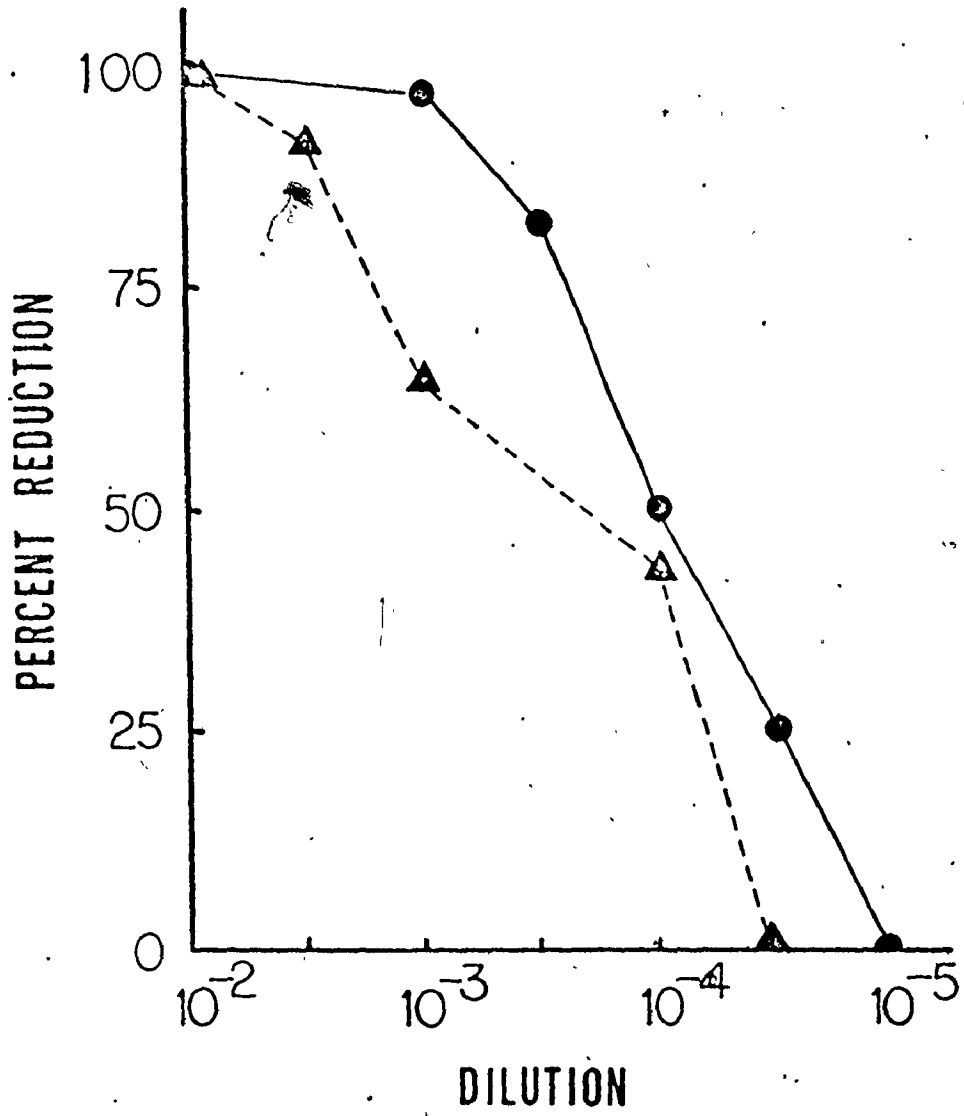


Figure 28

Interferon titrations on rabbit kidney cells. Dilutions of interferon prepared in MEM + 2% FCS were applied to monolayers of secondary rabbit kidney cells and incubated overnight at 37°C. The cells were then washed twice with warm HBSS and infected with 3×10^7 PFU of VSV. After one hour of virus adsorption, the cells were washed 3 times, MEM + 2% FCS was added and the cells incubated overnight. VSV titers were assayed on BHK-21 cells. Tissue culture interferon (●—●); Poly (I)-Poly (C) serum interferon (▲---▲).



overnight with tissue culture interferon or serum interferon. The cells were then washed, infected with vaccinia virus and the 24 hour yields of virus were determined. Tissue culture interferon did not suppress the replication of vaccinia virus in normal PE cells (Figure 29). Some reduction in virus yield was obtained with serum interferon, the 50% reduction titer being about 1:200 (Figure 29).

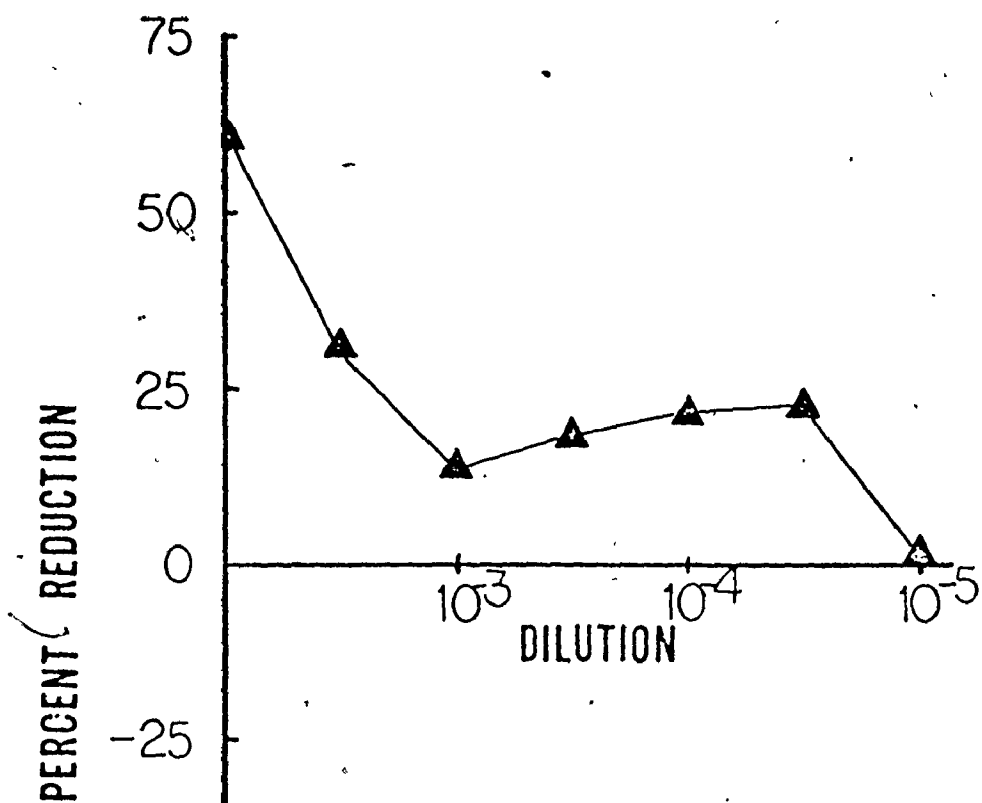
The effect of serum interferon on normal PE cells was further examined by treating the PE cells from three additional normal rabbits with a 1:10 dilution of the preparation. The PE cells were incubated overnight with the interferon containing serum or normal serum, infected with vaccinia virus and incubated overnight. The reductions of virus yield in the three sets of treated PE cells were 69%, 76% and 50%, respectively. Viral DNA synthesis in these cells, as determined by pulse labelling with ^3H thymidine at 5 hours after infection, was 40% less in the interferon treated cells than in control cells. Viral antigen could be detected in interferon treated and untreated PE cells by indirect immunofluorescence. Quantitation by complement fixation of viral antigen synthesized at 6 hours after infection revealed that serum interferon treatment reduced antigen synthesis by about 50% (Table 4).

VIII. Replication of Viruses Other Than Vaccinia Virus in Macrophages

Preliminary experiments were undertaken to determine the specificity of inhibition of virus replication by macrophages obtained from rabbits previously infected with vaccinia virus. Peritoneal exudate cells from normal rabbits were examined for their ability to support the replication of Sindbis virus, Semliki Forest virus, two strains of vesicular stomatitis virus and Pichinde virus. Only Pichinde virus was replicated in normal PE cells. As can be seen in Figure 30, Pichinde virus did not replicate in activated PE cells from a rabbit infected with vaccinia virus. As with vaccinia virus, Pichinde virus antigens were synthesized in activated macrophages although progeny infectious virus could not be detected. Thus, the activation of macrophages does not appear to be specific for the inducing virus and probably inhibits other viruses by the same mechanism as was found for vaccinia virus.

Figure 29

Interferon titrations on rabbit PE cells. Dilutions of interferon in MEM + 2% FCS were added to tubes containing 10^6 normal rabbit PE cells and incubated overnight at 37°C . The cells were washed, infected with 8×10^6 PFU of vaccinia virus for one hour and then washed three more times. Fresh medium was added to the tubes which were incubated overnight and then frozen. Triplicate tubes for each interferon dilution were pooled and assayed for virus titer on Vero cells. Tissue culture interferon (●---●); Poly (I)-Poly (C) serum interferon (▲—▲). These data are representative of duplicate experiments.



7.

TABLE IV

THE EFFECT OF SERUM INTERFERON ON VACCINIA VIRUS ANTIGEN SYNTHESIS IN
NORMAL PE CELLS¹

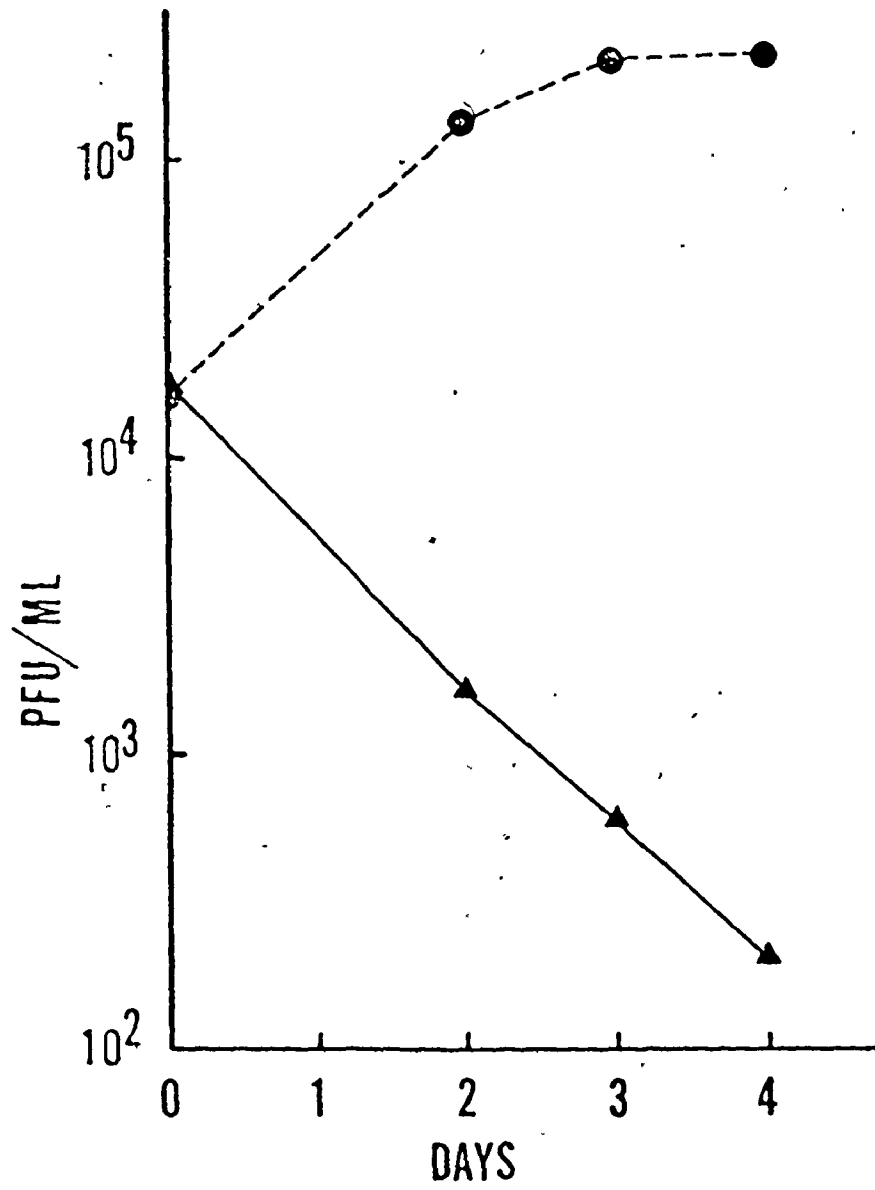
PE Cells from Rabbit No.	Titer of Complement-Fixing Antigen	
	PE Cells + Serum Interferon	PE Cells + Normal Rabbit Serum
1	1:16 ²	1:32
2	1:8	1:16
3	1:8	1:32

¹ 10^7 adherent PE cells were incubated overnight with a 1:10 dilution of either serum interferon or of normal rabbit serum, washed, and then infected with vaccinia virus at a MOI of 5. The cells were incubated for 6 hours, washed, and lysed in 0.5 ml of saline. The lysate was tested for CF activity using vaccinia antiserum.

² Titer represents dilution of antigen giving 50% endpoint in complement fixation test with 4 units of antiserum against vaccinia virus.

Figure 30

Growth of Pichinde virus in rabbit PE cells. Rabbit PE cells were infected with Pichinde virus at a multiplicity of 2 PFU/cell, for one hour. The cells were washed, resuspended at a concentration of 10^6 cells/ml and incubated at 37°C until sampled. Cells were lysed by freeze-thawing and the virus was titered on Vero cells. Normal PE cells (●---●); activated PE cells (▲—▲).



IX. Effect of Chemical Agents on the Growth of Vaccinia Virus in Rabbit Macrophages

It has been reported that certain chemical agents, namely trypan blue and hydrocortisone, are capable of abolishing the activated characteristics of mouse macrophages (Hibbs, 1974). The effect of various chemical agents on the ability of rabbit macrophages to either support or inhibit the replication of vaccinia virus was therefore examined in an attempt to abolish the inhibitory effect of activated macrophages on the virus. The chemical agents included trypan blue at 0.75%, 0.1%, 0.01%, .005%, .001%; hydrocortisone at 2.5×10^{-5} M, 5.0×10^{-5} M; benadryl at 10^{-3} M, 10^{-4} M; india ink at 0.1%, .01%; dimethylsulfoxide at 10%, 4%, 3%, 2%, 1% and antirabbit gammaglobulin at a 1/20 dilution. Various treatment regimens were performed with the cells which were then infected with vaccinia virus and growth curves of the virus performed. In general, the cells were pretreated with the chemical agents for 1, 2, 12, or 48 hours, infected with the virus and then further incubated both in the presence or absence of each specific chemical agent. In some experiments, the chemical agents were added simultaneously with the virus.

There was no change in the ability of the activated macrophage to inhibit the growth of vaccinia virus when the cells were pretreated for 0, 1, 2, or 48 hours with all of the above described chemicals. It was noted that the activated state of the macrophage changed when the activated macrophages were preincubated for 48 hours in MEM plus 10% FCS. The cells then underwent a morphological change from round to fibroblast like. When this happened, the cells could then support the replication of vaccinia virus.

DISCUSSION

The replication of vaccinia virus was blocked in activated macrophages. When peritoneal exudate (PE) cells from normal rabbits were infected with vaccinia virus at a multiplicity of infection (MOI) of 1, there was a 2-1/2 log increase in the virus titer after 48 hours. When activated PE cells were infected with the same amount of vaccinia virus, there was only a slight rise in virus titer from 1×10^3 PFU/ml to 3×10^3 PFU/ml. These experimental results concur with those reported by other investigators. Tompkins, Zarling, and Rawls (1970) were the first to note the inability of vaccinia virus to replicate within activated rabbit macrophages. Their data showed that vaccinia virus increased almost 2 logs in normal rabbit PE macrophages after 3 days incubation. There was no increase of virus titer in activated rabbit macrophages. Subsequent papers have reported a 2 to 2-1/2 log increase in vaccinia virus titer after 2 days incubation in normal rabbit macrophages with no increase in activated rabbit PE macrophages (Avila et al, 1972; Schultz et al, 1974). In all cases, the titer of vaccinia virus in activated macrophages remained stable during the 3 day incubation period. I feel that the constant titer of virus during the incubation period is due to the stability of the residual virus which remained in the medium after adsorption and washing. When infected PE cells were washed more extensively with 4 to 5 washes of HBSS, the initial titer of virus dropped 1/2 to 1 log and then remained stable during the incubation period. It was demonstrated in the one step growth curve that vaccinia virus is very heat stable when incubated in culture medium. There was no detectable loss of titer during the 18 hours of incubation.

Increasing the infecting dose of vaccinia virus to 20 PFU/cell did not overcome the block of virus replication in activated macrophages. Increasing the multiplicity of infection from 0.1 to 20 PFU/cell increased proportionally the amount of virus present at zero time.

The failure to show any production of infectious virus in activated macrophages inoculated with 20 PFU/cell also suggests that a large majority of the PE cells from an activated animal are activated since it can be assumed that at such a high multiplicity of infection, each cell would be infected. Variation in the inoculum given to normal PE cells was reflected in variation in the amount of virus at zero time, the total increase in virus titer, and the time needed for the growth curve to reach maximum levels. This was due to the finite number of cells which were available for infection within each tube. With a low inoculum, the initial amount of virus was low but the increase in virus titer was large (almost 4 logs) with the growth proceeding during the entire 72 hours. With a high inoculum, the virus titer at zero time was large, the increase in titer was only 1-1/2 log and a plateau in the growth curve was reached at an earlier time.

A comparison of the one step growth curve of vaccinia virus in PE cells and in Vero cells also indicated that optimum virus replication was not achieved in normal rabbit macrophages. In Vero cells, virus adsorption and burst size were greater than in normal macrophages and the appearance of new virus was two hours earlier. Comparison of vaccinia virus infected Vero cells with infected rabbit PE cells in later experiments confirmed that vaccinia virus replication in normal rabbit PE cells is not as efficient as in other cell types.

The replication of vaccinia virus in activated rabbit macrophages appeared to be blocked during or after late viral protein synthesis and before viral particle formation. All the stages in viral replication proceeding late protein synthesis appeared identical in activated and normal PE cells. The adsorption of vaccinia virus to activated rabbit PE macrophages was equal to or higher than that for normal PE macrophages. A higher rate of viral adsorption to activated macrophages can be explained by the presence of anti-viral antibody on the surface of cells taken from vaccinia virus immune rabbits. This surface antibody could facilitate viral adsorption. It is well documented that cytophilic antibody can be found on the surface of macrophages and it can be

assumed that a portion of this antibody taken from animals recently infected with vaccinia virus would be vaccinia virus specific. Koszinowski et al (1975) have reported the increased clearance of vaccinia virus by PE cells taken from vaccinia virus infected mice. PE cells from normal mice cleared 16% of the vaccinia virus inoculum, PE cells from mice injected once with vaccinia virus showed a 55% virus clearance and PE cells from mice injected 3 times with vaccinia virus cleared 88% of the available virus. Maximum clearance took place during the first hour of incubation.

The ability of activated macrophages to adsorb vaccinia virus has been previously reported. Avila et al (1972) reported that vaccinia virus adsorbed equally well to normal and activated PE macrophages. Adsorption was measured by titring cell associated infectious virus after 90 minutes of incubation. The discrepancy between my findings that activated macrophages adsorb slightly more virus than normal macrophages and Avila et al's results can be explained by the differences in methods used to detect virus adsorption. Detection by radioactively labelled virus is more sensitive than virus detection by plaquing and could detect small differences in the rate of adsorption between normal and activated macrophages.

The percentage of virus adsorption to the macrophages was between 10% and 35% of the virus inoculum. This was always much lower than the amount adsorbed to the Vero cells. Avila et al (1972) have reported a virus clearance of 40% by rabbit macrophages. Maximum adsorption of vaccinia virus to normal and activated macrophages as well as to Vero cells took place during the first 30 minutes of incubation. Rapid adsorption of vaccinia virus to HeLa cells and L cells has also been reported. Dales (1963) reported that 60% of vaccinia virus inoculum was adsorbed to L cells during the first 10 minutes of incubation. Joklik (1964a) reported that HeLa cells had adsorbed 45% of the vaccinia virus inoculum in the first 10 minutes of incubation.

Experiments with cells infected with radioactively labelled virus indicated that a significant amount of the virus eluted from the

cells during incubation. I found that even though the amount of virus which dissociated from activated PE macrophages was large, it was identical to the amount which dissociated from normal PE macrophages. Viral dissociation from the PE macrophages was much greater than that from infected Vero cells. After 5 hours incubation at 37°C, the level of viral dissociation was 55% in normal macrophages, 58% in activated macrophages, and 27% in Vero cells. Elution of Rabbitpox virus has been reported with HeLa cells (Joklik, 1964a). In that system, 15% of the cell associated virus had eluted after 6 hours of incubation.

Much of the eluted virus had been at least partially uncoated. This was detected by measuring the DNase susceptibility of the eluted virus. The greatest change in DNase susceptibility took place during the first hour of incubation. At this time, damage to the protective coat of the DNA had occurred in 58% of the virus which had eluted from normal PE cells, in 63% of the virus which had eluted from activated PE cells and in 40% of the virus which had eluted from Vero cells. This alteration in the DNase susceptibility of eluted virus was not found in similar experiments with rabbitpox virus infected HeLa cells (Joklik, 1964a). In that system, the eluted virus did not demonstrate a sensitivity to DNase. Alterations of eluted virus have been reported with other virus systems. Picornaviruses which elute from cells have lost a polypeptide and are unstable (Fenner *et al*, 1974). This has been explained as a first step in uncoating. A similar uncoating process has not been described for the Poxviruses. The most likely explanation for the DNase susceptibility of eluted vaccinia virus in the macrophage system, is that of another type of abortive infection. The virus enters the cell in a phagosome which fuses with a lysosome. This exposes the virus to lysosomal enzymes which disrupt a part of the viral coat. The digested virus is then released into the medium along with other digested material.

The uncoating of vaccinia virus occurred at normal levels in activated macrophages. This was first indicated by the drop in vaccinia virus titer seen in infected activated macrophages during the early

hours of incubation. This early drop in infectious titer, called the eclipse phase, indicated the release of viral DNA from its protective coat and its loss of infectivity under normal conditions.

The appearance of DNase susceptible viral DNA within activated macrophages confirmed that vaccinia virus was uncoated inside the cells. The uncoating began after one hour of incubation and proceeded at a steady rate until four hours after infection at which time it plateaued at approximately 50% of the virus uncoated. Vaccinia virus uncoated equally well in activated and normal macrophages and in Vero cells. The uncoating data of vaccinia virus in rabbit macrophages and in Vero cells is similar to that reported for other cell types. Joklik (1964a) reported the uncoating of rabbitpox virus in HeLa cells began at one hour of infection and continued rapidly for the next two hours. The lag period in DNA uncoating was dependent on the amount of infecting virus and with an inoculum of 90 PFU/cell, a peak of free viral DNA appeared by two hours after infection (Magee and Miller, 1968). The percent of viral uncoating varied for different cell lines but was never 100% (Joklik, 1964a). Rabbitpox virus underwent 67% uncoating in L cells and only 12% in KB cells.

The importance of vaccinia virus uncoating in activated macrophages was twofold. First, it indicated that the virus was successfully able to enter the cell and to release its nucleic acid which was then available for the next step in viral replication. The uncoating of vaccinia virus also indicated that some viral RNA and viral protein synthesis had taken place. A virally coded and virally produced uncoating protein is necessary for the second stage of vaccinia virus uncoating which is the release of viral DNA (Kates and McAuslan, 1967b). The presence of uncoated viral DNA is presumptive evidence that some viral RNA and protein has been synthesized and that the block in vaccinia virus replication in activated macrophages is not due to a complete block in viral transcription or viral translation.

Vaccinia virus was able to synthesize DNA in activated rabbit macrophages. The profiles for DNA synthesis in vaccinia infected normal

and activated PE cells were very similar. The profiles, which represented the total number of ^3H -thymidine counts per minute incorporated into TCA insoluble material, showed a rise at 2-1/2 hours. The peak of DNA synthesis dropped at 7 hours in both types of infected macrophages. The noninfected normal and activated PE cells showed only low background levels of DNA synthesis. DNA synthesis in the pellet fraction after cell lysis again showed a peak of DNA synthesis from 2-1/2 to 7 hours in both activated and normal PE cells infected with vaccinia virus. I believe that this peak of ^3H thymidine incorporation was due to vaccinia virus DNA synthesis for several reasons. Simultaneous experiments with uninfected PE cells never demonstrated a peak of DNA synthesis and always remained at background levels throughout the experiment. A similar peak of DNA synthesis was present in vaccinia virus infected Vero cells from 2 to 6 hours after infection and was absent in noninfected Vero cells. This peak was present in the cytoplasmic fraction of the Vero cell lysate. Studies by other investigators of vaccinia virus DNA synthesis have described a similar burst of DNA synthesis in vaccinia infected cells. Joklik and Becker (1964) reported a peak of DNA synthesis in vaccinia virus infected HeLa cells from 2 to 4 hours after infection when measuring both total DNA and cytoplasmic DNA. Peaks of DNA synthesis at 3 to 4 hours after vaccinia virus or rabbitpox virus infection have also been reported by Kates and McAuslan (1967b) and LaColla and Weissbach (1975). These investigators positively identified the DNA peaks as vaccinia virus DNA. Because the bouyant density of vaccinia virus DNA and HeLa cell DNA vary enough to separate on a CsCl gradient, it was possible to show that the peak of DNA in the vaccinia virus infected cells had a bouyant density similar to that of vaccinia virus (Joklik and Becker, 1964). The DNA present in the peak of DNA synthesis was also shown to hybridize with vaccinia virus DNA (LaColla and Weissbach, 1975). Unfortunately, in my experiments, the bouyant density of rabbit DNA is too similar to that of vaccinia virus DNA to be separated on a density gradient.

I was unable to demonstrate a significant peak of DNA synthesis

using only the cytoplasmic fraction from infected PE cells. I feel that this was due to an inability to thoroughly lyse the cells so that the cytoplasmic contents could be separated from the nuclei. Swelling with RSB and then dounce homogenizing is a standard procedure for cell lysis and worked well in the separation of the Vero cell fractions. This same procedure made the PE cells permeable to trypan blue. However, most of the newly synthesized DNA pelleted with the nuclei. Recent experiments have indicated that some specific DNA of vaccinia virus is synthesized in the cell nucleus (LaColla and Weissbach, 1975). Some of the DNA present in the pellet fraction could possibly have been produced in the nucleus. However, I feel that a large portion of it was synthesized in the cytoplasm and then trapped by the incompletely lysed cells and remained in the pellet fraction.

The synthesis of vaccinia virus DNA in activated macrophages provided more indirect evidence that vaccinia virus proteins were made within the cell. Continuous protein synthesis is required for vaccinia virus DNA replication (Joklik and Becker, 1964; Kates and McAuslan, 1967b). The addition of puromycin or cyclohexamide to infected cells inhibited the synthesis of viral DNA. Therefore, the synthesis of vaccinia virus DNA strongly suggested that viral proteins were also made.

The synthesis of specific proteins of vaccinia virus in activated macrophages was directly demonstrated by immunofluorescence, by immunodiffusion, and by polyacrylamide gel electrophoresis (PAGE). Vaccinia virus specific antigens were detected in both activated and normal macrophages by immunofluorescence. The pattern of immunofluorescence was identical in both cell types for the first 9 hours of infection. An equal proportion of activated and normal macrophages produced vaccinia virus specific antigen. All the positive cells possessed a dim diffuse fluorescence throughout the cytoplasm. The presence of fluorescence throughout the cytoplasm was probably due to multiple sites of infection within each cell. By 22 hours, the sites of antigen synthesis had enlarged and fused together so that the entire cell was filled with bright fluorescence. This phenomenon has been described by Cairns (1960).

Immunofluorescent studies of vaccinia virus infected activated and normal macrophages have also been described by Avila et al (1972). The number of infected cells increased in their normal macrophage cultures from 25% at 24 hours to 100% by 48 hours. There was no increase in the number of infected activated macrophages during the 72 hour incubation. They reported that less than 1% of the activated macrophages were producing antigen. The small number of antigen producing activated macrophages seen by Avila et al can be explained by the low infecting dose of virus they used. They infected their cells with an inoculum of 0.1 PFU/cell. Because no infectious virus was produced in the activated macrophage cultures, the infection was not amplified. The percentage of antigen producing cells remained the same as the percentage of cells initially infected, which was low. Because I used an inoculum of 2.5 PFU/cell, more cells were initially infected, and the number of antigen producing activated macrophages was increased.

The synthesis of vaccinia virus proteins in activated macrophages was also demonstrated by two dimensional immunodiffusion. At 6 hours after infection, three virus specific immunodiffusion bands were detected and by 9 hours four bands were present. These bands were determined to represent vaccinia specific antigens for several reasons. The antiserum used to detect the bands was prepared for its specificity to vaccinia virus by a procedure described by Cohen and Wilcox (1966). The procedure involved infecting rabbits with vaccinia virus intradermally and then injecting them with repeated doses of purified vaccinia virus intravenously. Secondly, the immunodiffusion bands produced using the antiserum were shown to be nonidentical to the two bands produced by fetal calf serum which is the major contaminant in most viral antisera. Thirdly, no immunodiffusion bands were present when noninfected rabbit macrophages were used as the antigen. Finally, the sequential appearance of the bands reflected the onset of viral protein synthesis and the accumulation in detectable amounts of viral proteins within the cell. At 0 and 2 hours after infection, no bands were present. The first bands were seen at 4 hours with an increase in band number at later

times. The sequential appearance of bands was also seen with infected normal macrophages and infected Vero cells.

The lysates of activated and normal macrophages infected with vaccinia virus gave a comparable number of bands (usually 4). Infected Vero cell lysates and infected PRK cell lysates gave 6 to 7 immunodiffusion bands. The greater number of bands in the later two cell lines can be attributed to the more productive infection possible in Vero and PRK cells. The vaccinia viral antigens detected by immunodiffusion are most probably soluble vaccinia antigens and not structural viral antigens. In the preparation of infected cell lysate used as antigen, the cells were lysed and then sonicated with no attempt to disrupt viral particles or virions in the process of morphogenesis. Westwood *et al* (1965) have reported that the strongest immunodiffusion bands formed with vaccinia virus infected cell extracts were virus soluble antigens and not structural antigens. They were able to produce a maximum of 17 immunodiffusion bands using an antigen extract of dermal pulp obtained from a rabbit infected with vaccinia virus. Only the minor immunodiffusion bands were shown to be identical to structural antigens of the virus.

The detection of 6 or 7 bands with vaccinia infected Vero or PRK cells is comparable to the results reported by other investigators. Cohen and Wilcox (1966), using an antigen prepared from vaccinia virus infected KB cells, were able to detect 7 bands by immunodiffusion. Extracts of cowpox virus infected rabbit skin or chorioallantoic membrane prepared by Rondle and Dumbell (1962) also yielded 7 immunodiffusion bands. Using similar techniques, Appleyard and Westwood (1964) were able to produce over 20 immunodiffusion lines with lysates of HeLa cells infected with rabbitpox virus. No explanation was given for the large number of bands they were able to detect.

The production of vaccinia viral proteins was also detected by SDS-PAGE. The majority of the polypeptide bands produced in infected activated macrophages was felt to be virus specific for several reasons. The sequential appearance of the gel bands after infection corresponded to the sequential synthesis of vaccinia viral proteins reported in the literature. Moss and Salzman (1968) studying vaccinia virus infected

HeLa cells have reported that early protein synthesis took place 1 to 3 hours after infection, intermediate protein synthesis was from 3 to 5 hours and late viral proteins were synthesized from 4 to 8 hours after infection. Holowczak and Joklik (1967) investigated the synthesis of structural proteins of vaccinia virus in L cells. They found that large amounts of structural proteins were made beginning at 4 to 5 hours after infection with the greatest amount made from 6 to 7 hours after infection. Pennington (1974) and Oppermann and Koch (1976) using slab PAGE of pulse labelled infected BSC₁ cells and HeLa cells have also demonstrated the sequential appearance of vaccinia viral proteins. A significant number of proteins were made at 2 hours after infection with the majority of the bands appearing after the onset of DNA synthesis. In my study, the sequential appearance of polypeptide bands in both the infected normal and infected activated macrophages was similar. Only a few faint bands were present during the first 30 minutes of infection. Many new bands appeared at 4 hours after infection with maximum protein synthesis at 6 and 7 hours after infection. The sequential appearance of protein bands in the virus infected macrophages lagged somewhat behind the reported appearance of polypeptide bands in the HeLa, L and BSC₁ cells and in my own experiments with Vero cells. This was again probably due to the less productive type of infection in macrophages as compared to the other cell lines. The appearance of significant new gel bands at 4 hours after infection paralleled the appearance of virus specific immunodiffusion bands detected in similarly infected macrophages.

The similarity in gel profiles between the infected activated and infected normal macrophages demonstrated that many of the same polypeptides were synthesized within the cells infected with vaccinia virus. Examination of the infected cell lysates from two activated rabbits and three normal rabbits at 6 hours after infection revealed that 25 polypeptide bands were similar. Several differences were also noted. There were three polypeptide bands in the infected activated macrophages which were not present in the infected normal macrophages and one band in the infected normal macrophages which was absent in the infected activated

cells. A comparison of the gel profile of vaccinia virus infected Vero cells at 4 hours after infection with the profiles of the infected macrophages at 6 hours after infection also revealed that the major bands synthesized were present in all three cell types. Finally, the dissimilarity between the gel profiles of virus infected and noninfected activated macrophages and normal macrophages was further proof that the bands present in the infected cell lysates were vaccinia virus specific.

Examination of infected macrophage lysates which had been pulsed with ^3H amino acids and then chased with cold medium revealed one difference between activated and normal macrophages. A shift in band position at 6.5 cm from the top of the gel was noted in both the activated and normal macrophages during the chase period. I am uncertain, however, whether to attribute this shift in band position to post-translational modification of the polypeptide or to a slight irregularity in the gel. The band shifted upward during the normal macrophage chase period and shifted downward during the activated macrophage chase period. The position of the band in the normal macrophages after 6 hours of chase was identical to that in the activated macrophages after no chase. These two samples were run in the center lanes of the gel. Reports by other investigators on the position of vaccinia virus polypeptides which underwent post-translational cleavage do not reveal any major changes in this region of the gel. Other investigators have reported that the concentration of bands P4a and Px or P4b diminished during the chase period while band 4a appeared (Katz and Moss, 1970a; Pennington, 1973; Moss and Rosenblum, 1973; Pennington, 1974). These bands are between 60,000 and 100,000 dalton molecular weight and correspond to the band at 1.6 cm from the top of the gel in the Vero cell lysate. The other major post-translational change noted was the appearance of a new band near the bottom of the gel. This was designated either protein 9 (Pennington, 1973) or protein 10 (Moss and Rosenblum, 1973) depending on the investigator. This new polypeptide also appeared in the Vero cell lysate at 7.8 cm from the top of the gel.

A summary of the data on vaccinia virus protein synthesis indi-

cates that many vaccinia proteins are produced in activated macrophages. If the synthesis of individual polypeptides are examined in activated and normal macrophages, it appears that there is a difference in the synthesis of at least four polypeptides. This difference could be caused by the complete lack of synthesis of a polypeptide, by the aberrant synthesis of the polypeptide or by the modification of the polypeptide immediately after its translation. The absence of change in the pulse chase experiment suggests that the variation in polypeptides is probably not due to post-translational modification. However, with the data available, the significance of the small differences observed in polypeptide synthesis remains unclear.

The most obvious distinction between the activated and normal rabbit macrophages was found in the assembly of vaccinia virions in the infected cells. None or very few vaccinia virus particles were assembled in activated rabbit macrophages. This was best demonstrated with the sucrose density gradients using ^3H thymidine labelled infected cell lysates. At 22 hours after infection in activated macrophages, there was only a slight bulge in the region where vaccinia virus banded. This indicated the lack of accumulation in activated macrophages of DNA containing particles with the size and density of the virus. This was confirmed by the complete lack of vaccinia virus complement fixing antigen within the gradient. In opposition to this was the presence of large peaks of DNA containing particles in infected normal macrophages and infected Vero cells. The large peak of DNA containing material in normal macrophages at 22 hours after vaccinia virus infection corresponded with the presence of virus specific complement fixing antigens in the gradient. The vaccinia virus antigen appeared to be assembled in vaccinia virion particles and smaller sized material as it was present throughout the top two thirds of the gradient. The absence of either DNA containing particles or virus antigen within the gradient at 8 hours after infection indicated that the assembly of vaccinia virions took place at a reduced rate in normal macrophages when compared to other cell types. In infected Vero cells, a large peak of DNA containing vaccinia virus

particles was present at 8 hours after infection. By 22 hours, synthesis had stopped and all the particles within the cells had apparently been degraded.

The results of my experiments on the fate of vaccinia virus in activated rabbit macrophages have demonstrated that the virus was able to enter the macrophage and successfully uncoat in preparation for viral replication. Vaccinia virus DNA and protein were synthesized within the activated macrophage. Many of the same polypeptides were made in both vaccinia virus infected activated and normal macrophages; however, at least four differences in individual polypeptides were noted. There was a complete lack in production of infectious vaccinia virions with no apparent assembly of the virally synthesized DNA and protein into viral particles.

I have two postulates for the lack of infectious vaccinia virions in activated rabbit macrophages. It is possible that lysosomal enzymes or other antimicrobial molecules enclosed within the activated macrophages could digest the virus particles during or immediately after morphogenesis. Activated macrophages possess elevated levels of lysosomal enzymes (Pantelone and Page, 1975) which might be readily accessible to newly synthesized virus particles. Kochan and Golden (1974) have reported that heptane-extractable fractions from activated macrophages had anti-microbial activity while extracts from normal macrophages had no effect. The anti-bacterial factors were soluble in heptane, neutralized by albumin and thought to be free fatty acids. I have no data to support the theory that increased concentrations of antiviral factors within activated macrophages are responsible for their anti-vaccinia virus activity. Incubation of vaccinia virus with a 10% suspension of activated or normal macrophage lysates revealed no differences between the lysates from the two cell types in their ability to destroy the virus.

Pretreatment of activated macrophages with hydrocortisone or trypan blue did not abolish the cells inhibitory effect on vaccinia virus. Trypan blue is an inhibitor of lysosomal enzyme activity and

hydrocortisone is a membrane stabilizer which interferes with the transfer of lysosomal markers (Hibbs, 1974). Hibbs (1974) has reported that pretreatment of activated macrophages with either hydrocortisone or trypan blue inhibited the cytotoxic effect of activated macrophages against tumor cells. Morahan and Kaplan (1976) have reported that trypan blue reduced the cytotoxicity of activated macrophages to tumor cells but did not completely abrogate it. Dimitriu (1976) has reported that corticosteroids prevented activation of macrophages by lymphocyte supernatants but had no effect if given during or after macrophage arming. The lack of effect of either trypan blue or hydrocortisone in my experiments suggests that lysosomal enzymes may not act directly on vaccinia virus within the activated macrophage.

The lack of infectious virus within activated macrophages may also be due to the failure of the virus to produce all the necessary components needed for virion assembly. The profiles of SDS-PAGE gels have revealed several differences between polypeptide synthesis in activated and normal macrophages infected with vaccinia virus. These differences in polypeptides may be enough to prevent assembly of the virus particle and maturation into new infectious virions. I have no data to suggest a mechanism by which the activated macrophages could modify the synthesis of selected viral proteins. Further work concerning the synthesis of vaccinia viral proteins and the fate of the proteins after synthesis is needed to ascertain whether this is the mechanism of action of activated macrophages. Preliminary experiments with Pichinde virus suggested that it too was able to produce viral proteins within the activated macrophages while unable to produce infectious virus. Further studies with other viruses would be helpful to demonstrate the universality of the effect of activated macrophages on viruses.

Interferon is a viral inhibitory substance first described by Isaacs and Lindenman in 1957. It consists of a family of proteins coded for by cells after coming in contact with an interferon inducer, usually a foreign nucleic acid. Interferon is capable of inhibiting the replication of viruses without interfering with the synthesis of cellular

molecules. The inhibitory effect of interferon is species specific but not cell type specific. The sensitivity of viruses to interferon varies with the virus and cell system used. Vaccinia virus has been shown to be most sensitive to mouse and hamster interferon and less sensitive to human, rabbit, and bat interferon (Stewart et al, 1969). Variation in interferon susceptibility can be found within the Poxvirus group. The replication of both the WR strain of vaccinia virus and cowpox virus was completely inhibited in interferon treated chick embryo fibroblasts (Jungwirth et al, 1972). Interferon treated L cells, however, yielded incomplete inhibition of cowpox virus but complete inhibition of vaccinia virus. In general, vaccinia virus displays a reduced sensitivity to interferon. Rabbit interferon produced in cultured cells was about 250 times less inhibitory to vaccinia virus than to vesicular stomatitis virus, Semliki Forest virus, and sindbis virus (Stewart et al, 1969).

Although newcastle disease virus (NDV) has been the most widely used inducer of interferon, other molecules are capable of inducing interferon both in animals and in cultured cells. Interferon inducers effective both in vivo and in vitro are various DNA and RNA containing viruses, naturally occurring double stranded RNA and synthetic helical polyribonucleotides (Fenner et al, 1974). Interferon inducers effective in vivo only are bacterial endotoxins, whole protozoan and bacteria and synthetic polyanions.

Two types of interferon are now known to exist. The standard, well studied interferon (type I) is made by many cell types in response to a variety of inducers as described above. Immune interferon (type II) is produced by white blood cells during an immune response in vivo or by in vitro incubation of white blood cells with NDV, with various mitogens, with anti-lymphocyte serum, or in a mixed leucocyte culture (Notkins, 1975). Production of immune interferon is enhanced in sensitized animals or sensitized cells. Type I and type II interferon differ antigenically and in their stability to low pH. Type I interferon is stable at pH 2 while type II interferon is unstable at pH 2.

The mechanism of action of interferon has been widely studied in

vaccinia virus infected cells. It appears that interferon acts by selectively inhibiting vaccinia virus specific translation (Bodo et al, 1972) possibly through the action of a newly formed protein (Fenner et al, 1974). In interferon treated cells, early vaccinia virus RNA is synthesized but no early viral protein or viral DNA is made. Using interferon prepared in NDV infected L cells, Joklik and Merigan (1966) pretreated L cells with interferon for 16 hours and then infected them with vaccinia virus. They recorded an enhanced burst of early RNA synthesis but no production of viral DNA polymerase or synthesis of vaccinia virus DNA or late RNA (Joklik, 1968a). Metz and Esteban (1972) have reported an increased synthesis of vaccinia early RNA in interferon treated L cells with a complete block in virus polypeptide synthesis as detected by PAGE gel bands. A reduction in vaccinia virus protein synthesis in interferon treated L cells has also been demonstrated by the decrease in immunodiffusion bands measured at 2 to 4 hours after virus infection (Bodo et al, 1972). Interferon treatment of vaccinia virus infected CEF cells has yielded similar results. Early viral RNA synthesis was stimulated but vaccinia virus early protein synthesis was inhibited as evidenced by the disappearance of almost all the protein bands seen on a PAGE gel of lysates of pulse-labelled infected cells (Esteban and Metz, 1973). The reduction in viral polypeptides by interferon treatment was seen as early as 20 minutes after infection. A delay and reduction of vaccinia virus thymidine kinase synthesis, DNA polymerase synthesis, and DNA synthesis were also seen in interferon treated CEF (Levine et al, 1967). The stimulation of early viral RNA synthesis in interferon treated cells has been attributed to the lack of synthesis of a regulatory protein (Metz and Esteban, 1972).

As expected when no viral proteins were produced, interferon pretreatment of cells inhibited the complete uncoating of vaccinia virus (Magee et al, 1968). Vaccinia virus was able to undergo the first step of uncoating or core formation which is performed by the cell. The second stage of uncoating which is the release of viral DNA did not take place. This second uncoating stage is dependent on the synthesis of a

virally coded uncoating protein (Kates and McAuslan, 1967b).

After his early work had demonstrated that vaccinia virus RNA but no vaccinia protein was synthesized in interferon treated cells, Joklik (1968b) postulated that pretreatment with interferon prevented the attachment of viral mRNA to ribosomes. This was recently confirmed by Metz, Esteban, and Danielescu (1975). Sucrose gradients of interferon treated L cells infected with vaccinia virus demonstrated that the newly synthesized viral mRNA-protein complex was not associated with ribosomal 40s subunits. They suggested that this disaggregation of polyribosomes was due to the failure of the small ribosomal subunits to attach to the viral mRNA. It is not known how the cellular subunits distinguish the viral mRNA from the cellular mRNA. Metz's et al data also demonstrated that chain elongation in interferon treated cells was also inhibited. However, they stated that protein synthesis initiation was inhibited more than chain elongation in vaccinia virus infected interferon treated cells.

My data indicates that standard or type I interferon was not involved in the inhibition of vaccinia virus replication by activated rabbit macrophages. Pretreatment of normal rabbit macrophages with interferon produced in rabbit kidney cells had no inhibitory effect on the replication of vaccinia virus. Even using a high concentration of interferon because of the reported low sensitivity of vaccinia virus to interferon (Stewart et al, 1969) did not inhibit the replication of vaccinia virus in interferon pretreated macrophages. All the experimental data on the fate of vaccinia virus in activated rabbit macrophages supports the theory that the virus was able to uncoat within the activated macrophage and produced normal levels of viral DNA and many viral proteins. This is in direct conflict with the reported fate of vaccinia virus in interferon pretreated cells. Interferon treatment of L cells and HeLa cells inhibited vaccinia virus uncoating (Magee et al, 1968), viral protein synthesis (Joklik, 1968b; Metz and Esteban, 1972; Bodo et al, 1972; Esteban and Metz, 1973) and viral DNA synthesis (Levine et al, 1967; Joklik, 1968b).

The involvement of immune interferon (type II) in the inhibition of vaccinia virus replication in activated macrophages is less clear. Pretreatment of normal macrophages with a 1/10 dilution of serum from a poly I-C injected rabbit caused a 65% reduction in vaccinia virus titer. It is not possible to state what was responsible for the reduction in virus titer since unpurified rabbit serum was used to pretreat the cells. The inhibitory factor present in the poly I-C serum could have been type II interferon or a factor capable of activating macrophages. It is possible that the poly I-C induced various lymphocyte mediators, including macrophage activating factor, which were released into the bloodstream. Alexander and Evans (1971) have reported the poly I-C was capable of activating macrophages. Double-stranded RNA, either synthetic or natural, was shown to activate mouse macrophages both in vivo and in vitro. Activation was measured by nonspecific cytotoxicity to tumor cells. Injections of poly I-C have also enhanced the chemotactic responses of mouse macrophages to a level comparable to that in BCG activated macrophages (Meltzer et al, 1975).

The reduction in levels of viral DNA and viral protein synthesis in the macrophages treated with serum interferon tends to suggest a possible interferon-like inhibition. Along with the 65% reduction in virus titer, viral DNA synthesis was reduced by 60% and viral protein synthesis was lowered by at least 50% as measured by complement fixation. It was not possible to determine whether the reduced number of immunofluorescent positive cells in the serum interferon culture was due to a low number of infected cells which were producing virus or was due to the inability of the antigen producing cells to produce infectious virus which would amplify the infection. Reported studies with type I interferon treated cells have demonstrated that the drop in vaccinia virus titer was accompanied by a concomitant reduction in viral DNA synthesis and viral protein synthesis. We have to assume that the type of interference caused by type I and type II interferons are similar because there have been no reports on the mechanism of action of type II interferon. The reduction of vaccinia virus titer in activated macrophages

was not accompanied by a reduction in viral DNA or most viral proteins. Therefore, vaccinia virus replication in the macrophages treated with serum interferon was probably inhibited by an interferon type of action and not by the same type of mechanism seen in macrophages taken from activated animals.

The production of various mediators or lymphokines by stimulated lymphocytes is a complex phenomenon. It is quite probable that a number of lymphocyte products are produced which have yet to be described. It is possible that the same molecule may cause multiple responses in a cell and that similar functions may be performed by different molecules. The production during an immune response of a type II interferon like molecule in association with other lymphokines has been reported (Salvin et al, 1973). The interferon activity was associated with macrophage migration inhibition factor activity in serum from mice given old tuberculin or BCG cells intravenously. Both activities had a similar time course of appearance, both were unstable at pH 5 and both were present in the same exclusion peak from Sephadex G-100. The inhibitory property of interferon has also been associated with the enhancement of phagocytosis by macrophages (Huang et al, 1971; Donahoe and Huang, 1973; Donahoe and Huang, 1976). The enhancing factor was stable at pH 2, inactivated by trypsin and did not sediment at 100,000 xg (Huang et al, 1971). Because of the multitude of factors which could have been present in the serum of the rabbit injected with poly I-C, it is not possible to positively attribute the inhibition of vaccinia virus replication to either interferon type II or to a macrophage activating factor without further studies using purified factors.

SUMMARY

The data derived from my studies indicate the following:

1. In normal rabbit macrophages, vaccinia virus was able to produce new infectious virus, while activated rabbit macrophages were capable of blocking the replication of vaccinia virus.

2. Vaccinia virus was able to adsorb to, penetrate, and uncoat within the activated macrophages. Levels of adsorption and DNA uncoating in activated macrophages were equal to those in normal macrophages.

3. Vaccinia virus DNA was synthesized within activated macrophages. The amount of tritiated thymidine incorporated into acid insoluble material after vaccinia virus infection was comparable in both activated and normal macrophages.

4. Vaccinia virus specific proteins were synthesized in activated macrophages. Detection by immunofluorescence and immunodiffusion indicated that similar amounts of viral protein were made in activated and normal macrophages. Examination of specific polypeptide synthesis by SDS-PAGE indicated that there were at least three polypeptides present in virus activated macrophages infected with vaccinia virus which were absent in the virus infected normal macrophages and at least one polypeptide present in the virus infected normal macrophages which was absent in the virus infected activated macrophages.

5. No virus particles with the size and density of vaccinia virions were detected in lysates of activated macrophages infected with vaccinia virus.

6. Vaccinia virus infection of normal rabbit PE macrophages was not as productive as infection in Vero cells. This included all phases of the reproductive cycle including adsorption, DNA synthesis, protein synthesis, and particle formation.

7. Pretreatment of normal rabbit macrophages with interferon type I (classical type) did not block the replication of vaccinia virus.

Pretreatment of normal rabbit macrophages with serum from poly I-C injected rabbits reduced the replication of vaccinia virus but the characteristics of the abortive infection appeared different than in activated macrophages.

8. The observations suggest that the inhibition of replication in activated macrophages is mediated by mechanism(s) unique from that induced by interferon.

APPENDIX I

SOLUTIONS

Agarose for Immunodiffusion	
Agarose (Sigma, purified for electrophoresis)	1 gm
Borate buffered saline, pH 8	100 ml
Sodium azide (Eastman)	0.1 g
Borate Buffered Saline, pH 8	
Borate buffer stock	1 part
Saline	19 parts
Borate Buffer Stock	
Boric acid	6.184 g
Borax (Na tetraborate)	9.536 g
Na Cl	4.384 g
H ₂ O, distilled	500 ml
Coomassie Blue Stain for Immunodiffusion	
Coomassie blue	0.25 g
Immunodiffusion destain buffer	100 ml
FAA Fixative	
70% ethanol	20 parts
Formalin	2 parts
Acetic acid	1 part
Immunodiffusion Destain Buffer	
Methanol	45 ml
Acetic acid	10 ml
H ₂ O, distilled	45 ml
Phosphate Buffered Saline (PBS)	
NaCl	40 g
KCl	1 g
Na ₂ PO ₄	13.9 g
KH ₂ PO ₄	1 g
H ₂ O, distilled	5000 ml
Adjust pH to 7.4	
Reticulocyte Standard Buffer (RSB)	
NaCl	.01 M
MgCl ₂	.0015 M
Tris, pH 7.4	.01 M

Saline	
NaCl	0.85 g
H ₂ O, distilled	100 ml
Scintillation Fluid	
Toluene, scintillation grade	1 gallon
Fluoralloy Dry Mix Formula TLA (Beckman)	32.2 g
(8 g/l PBD, 0.5 g/l PBBO)	
Slab Gel Sample Preparation Buffer	
Tris, pH 6.8	0.05 M
Sodiumdodecylsulfate	1%
2 mercaptoethanol	1%
Glycerol	10%
Slab Gel Destain	
Methanol	25%
Acetic Acid	7%
H ₂ O, distilled	68%
Slab Gel Stain	
Coomassie brilliant blue R-250	0.25 g
Slab gel destain	100 ml

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