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INFLUENCE OF GLUTAMINE AND GLUCOSE
UPON HERPES SIMPLEX VIRUS PRODUCTION
BY HELA CELLS.

The University of Oklahoma, Ph.D., 1961
Bacteriology

University Microfilms, Inc., Ann Arbor, Michigan

THE UNIVERSITY OF OKLAHOMA
GRADUATE COLLEGE

INFLUENCE OF GLUTAMINE AND GLUCOSE UPON HERPES SIMPLEX
VIRUS PRODUCTION BY HELA CELLS

A DISSERTATION
SUBMITTED TO THE GRADUATE FACULTY
in partial fulfillment of the requirements for the
degree of
DOCTOR OF PHILOSOPHY

BY
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Oklahoma City, Oklahoma

1961

INFLUENCE OF GLUTAMINE AND GLUCOSE UPON HERPES SIMPLEX
VIRUS PRODUCTION BY HELA CELLS

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ACKNOWLEDGMENT

The author wishes to express his gratitude to Dr. L. Vernon Scott for the aid and encouragement that made this investigation possible. Thanks are extended to the entire faculty of the Department of Microbiology for guidance and inspiration.

Acknowledgment is made to National Institutes of Neurological Diseases and Blindness Grant B 964, to National Institutes of Health, General Medical Sciences Division Grant GF-11,145 and to the Brown Hazen Fund of the Research Corporation for support of this investigation.

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CHAPTER I

INTRODUCTION

Alterations in cellular metabolism that occur as a result of infection of the cell by a virus are imperfectly understood. Elucidation of metabolic changes caused by the primary event of viral infection may lead to expression in biochemical terms of the mechanisms by which a cell accomplishes the synthesis of specific protein in general. In addition, effective chemotherapy for viral infections may result from precise knowledge of the reactions by which viruses are proliferated intracellularly.

Studies of the effects of viral infections on cellular metabolism have been made by employment of tissues from animals infected in vivo, of tissue fragments cultivated and infected in vitro, and of cell cultures propagated and infected in vitro. Changes in cellular constituents and products relative to time

following infection have been described in certain studies. Other investigations have determined the effect on the production of virus of controlled variation in the nutrients available to the cell.

Metabolism of Tissue Infected by Virus In Vivo

Before techniques were developed for prolonged maintenance of animal cells in vitro, tissues from infected animals were utilized in studies of the biochemical effects of viral infection on cellular economy. An example of the early work with tissues infected in vivo may be found in the report of Crabtree in 1928. With the use of manometric techniques he was able to demonstrate a high rate of aerobic glycolysis in slices of fowlpox infected tissue which is typical of malignant tissue. He reported further that such altered carbohydrate metabolism occurred with vaccinia lesions of chickens, but not of rabbits. Crabtree found Rous sarcoma cells to possess the metabolism characteristic of tumors. No alteration was noted in the carbohydrate metabolism of brain tissue of guinea pigs which were moribund from rabies at the time of sacrifice. However, there was no claim by the author that any of the changes observed were more than indirect effects of viral infection.

Barron (1932) noted that the succinodehydrogenase activity of rabbit brain was reduced by in vivo infection with herpes simplex virus. In contradistinction to normal tissue, the lesions of Rous sarcoma in chickens and of infectious myxoma in rabbits exhibited no succinodehydrogenase activity.

Definition of the primary effects of infection upon host cells in chemical terms is almost impossible to achieve by use of such in vivo infection. Observations are complicated by secondary inflammatory changes of the host as well as by products of destroyed cells. Further, any changes which occur in the metabolism of the few infected cells of the animal are obscured by the preponderance of uninfected cells that are present.

The difficulty of obtaining exact information of the cellular chemistry of viral infection by studies of tissues infected in vivo may be illustrated by the polemics among several reputable investigators concerning the primary metabolic effects of infection with poliomyelitis virus. Brodie and Wortis (1934) found the respiratory quotient of spinal cord and brain from poliovirus-infected monkeys to be identical with that from normal animals. Backer and Kabat (1942) reported that the anaerobic glycolysis in brain tissue of

mice which had been infected in vivo with poliomyelitis virus was inhibited by 15 percent. These workers found also that the amount of inhibition was directly proportional to the degree of encephalitis developed by the animals before sacrifice. However, Wood (1944) and Wood et al. (1945) were unable to confirm these findings. Furthermore, the latter investigators suggested that even if differences had been shown in the activities of tissue from infected and normal animals, such differences could have been due to secondary effects of infection. For example, infected mice lose weight. Nevertheless, Racker and Krimsky (1946) reported reduced glycolysis in brain tissue of animals which had been infected in vivo with poliomyelitis virus or mouse encephalomyelitis virus. Specifically, conversion of fructose-6-phosphate to fructose-1,6-diphosphate was said to be inhibited.

The techniques employed in such investigations were questioned seriously by Utter et al. (1945). They reported that intracellular enzymes were released during preparation of the tissue for in vitro study. These enzymes destroyed diphosphopyridine nucleotide, a coenzyme necessary for glycolysis. Therefore, it was suggested that the validity of results obtained was uncertain.

Metabolism of Tissue Infected by Virus In Vitro

The development by Harrison (1906) of a method of tissue culture offered a system for investigation of the effects of viral infection at the cellular level which was more suitable than those employing intact animals. Tissue fragments cultivated in vitro are free of complicating secondary reactions which occur when the entire animal is infected. Also, more exact definition and closer control of the physical and chemical environments may be achieved in vitro than in vivo. Therefore, the extensive use of tissue cultures in studies concerning the biochemistry of viral infection is not surprising.

Victor and Huang (1944) showed that utilization of metabolic pathways in chick embryo fragments was altered by exposure to Western equine encephalomyelitis virus. The rate of glycolysis increased on the sixth day of infection, a time when it decreased in normal culture.

A communication by Thompson (1947) described the effects of several metabolites, metabolic antagonists, and enzyme inhibitors on the replication of vaccinia virus in chick embryo fragments that were cultivated in vitro. He reported that the addition of intermediary metabolites of the citric acid cycle to a simple salt solution did not affect the amount

of virus produced. However, cyanide, azide, and malonate did decrease viral production by the cells. Thompson and Wilkin (1948) found later that thienylalanine reduced viral proliferation in this system and that the inhibition was reversed specifically by phenylalanine. Thus, by employment of infected tissue fragments, it was demonstrated successfully that certain enzymes and at least one amino acid were essential for the production of vaccinia virus.

Pearson and Winzler (1949) and Rafelson et al. (1949) showed the influence of infection with Theiler's mouse poliomyelitis virus upon the metabolism of minced one-day-old mouse brain. Although rates of glucose disappearance and of lactic acid appearance were the same in infected and normal tissue, infection caused the incorporation of carbon into cellular fat to decrease and into protein to increase. In addition, infection stimulated incorporation of labeled phosphorus into ribonucleic acid by the cells. Significantly, this stimulation of nucleic acid metabolism was most marked during the period of maximal production of virus. Pearson (1950) employed the same tissue fragment-virus system to learn that several enzyme inhibitors decreased the yield of virus.

Identification of the metabolic pathways which furnish the energy for production of myxoviruses has been attempted.

Ackermann (1951a) studied influenza virus-infected fragments of developing chick embryo chorioallantoic membranes which were cultivated in vitro. Manometric determinations revealed no difference in oxygen uptake between infected and non-infected fragments. Malonate, an inhibitor of the tricarboxylic acid cycle, decreased production of virus by the cells proportionally to the suppression of respiration by this compound. Since malonate was not viricidal, did not interfere with infection of the cells, and caused no injury to the cells which prevented them from synthesizing virus after the inhibitor had been removed, Ackermann decided that the energy for production of virus was derived from oxidative phosphorylation. This conclusion was justified by demonstrations (Ackermann and Johnson, 1953; Eaton and Perry, 1953) that dinitrophenol, which uncouples oxidative phosphorylation, decreased viral production also. An even greater suppression of viral replication resulted from use in this system of another uncoupler of oxidative phosphorylation, 3,5-diiodo-4-hydroxybenzoate (Eaton et al., 1953).

Studies of the metabolism of the myxovirus-cell complex have not been restricted to determination of energy requirements for viral production. Ackermann (1951b) showed that methionine was necessary for growth of influenza virus

in cultures of chick embryo chorioallantoic membrane. Inhibition by methoxinine, an analogue of methionine, was reversed by the amino acid. Eaton et al. (1951) utilized cultures of chick embryo tissue to demonstrate suppression of multiplication of influenza and mumps viruses by basic amino acids. The concentrations of the amino acids employed exerted no detectable injurious effect on the tissue. Cushing and Morgan (1952) established the pyridoxine and thiamine requirements for production of these two myxoviruses in chick embryo tissue culture. Viral growth was inhibited by concentrations of analogues of these vitamins which had no discernable effect on the tissue per se.

The same tissue culture system was used by Levine et al. (1956) to study effect of several metabolites on the production of influenza virus. Although addition of glucose to a balanced salt solution supported the multiplication of both WS and NWS strains of this virus, only the WS strain replicated when pyruvate was the sole energy source of the cells. Attempts to explain the difference in nutritional requirements were unsuccessful. Burr et al. (1954), however, were unable to show any effect of the medium upon production of influenza and mumps viruses in this tissue culture system. Since the amount of virus was unchanged by

maintenance of the cultures in a balanced salt solution for as long as six days after infection, the investigators postulated that endogenous nutrients were more important than exogenous ones.

Methionine was demonstrated (Brown and Ackermann, 1951) to be necessary for production of poliovirus by minced human embryo brain tissue. Inhibition of viral replication by an analogue of this compound was reversed by presence of the metabolite. Brown (1952) employed cultures of minced monkey testicle to define metabolic requirements for growth of this virus. Methionine, phenylalanine, adenine, guanine, and uracil appeared to be essential. Franklin et al. reported in 1952 that fragments of human embryo brain and spinal cord infected in vitro with poliovirus utilized less glucose than did comparable normal cultures.

Considerable information concerning factors related to the formation of psittacosis virus in vitro has resulted from a continuing investigation by Morgan. He (1952a) showed that no virus was formed in cultures of minced chick embryo which were nourished with ox serum ultrafiltrate in balanced salt solution unless pteroylglutamic acid were available. This became apparent from the reversal by the growth factor of the inhibition of viral production caused by an analogue,

4-amino-pteroylaspartic acid. Using this cell-virus system, Morgan (1952b) studied the role of purines and pyrimidines in the synthesis of virus. Benzimidazole suppressed viral multiplication completely, although the culture produced virus when the compound was removed. Inhibition of viral proliferation by 2,6-diaminopurine was reversed readily by adenine, while inhibition by 8-azaguanine was nullified by guanine. Morgan (1952c) found also that thiouracil suppressed replication of virus in the cultures without affecting the tissue directly. Hare and Morgan (1954) demonstrated that the yield of psittacosis virus by such cultures in a balanced salt solution was increased by the presence of beef embryo extract or lactalbumin hydrolysate. This enhancement was not due to multiplication of the cells, since the same effect was observed in the presence of the mitotic inhibitor, colchicine. It was also demonstrated (Morgan, 1954) that phenylalanine, methionine, tryptophane, and pantothenic acid were necessary for viral growth in these cultures. Presence of appropriate analogues of these compounds at levels non-toxic to the tissue suppressed multiplication of virus.

Morgan (1956) published results of further experiments elucidating the relationship of cell nutrition to the initiation

of growth of psittacosis virus. If chick embryo fragments were maintained in a simple balanced salt solution for 13 days before infection, the cells were no longer capable of producing virus unless the solution was enriched with nutrients such as those found in embryo extract. However, a delay of 13 days after infection before addition of the extract did not prevent subsequent production of virus. Virus could be detected neither in the medium nor associated with the cells during the period between infection and the addition of the extract. Therefore, the author suggested that changes in the nutritional state of cells may activate latent infection in vivo. Since the chemical composition of the embryo extract which had been used as a nutrient in previous studies was not known, Johnson and Morgan (1956) attempted to determine more exactly the nutritional requirements of the cells for synthesis of psittacosis virus. They demonstrated first that a completely defined medium functioned as well as the extract in stimulation of production of virus. The investigators examined next the effect of deletion of specific compounds from this defined medium on viral replication. They showed that the fat soluble vitamins made no contribution to viral production, but that water soluble vitamins and amino acids were required. Coenzyme A and pyridine

nucleotide coenzymes did not stimulate viral replication.

Burney and Golub (1948) observed that among a number of enzyme inhibitors tested, only o-iodosobenzoate, hydroquinone, and iodoacetamide inhibited production of psittacosis virus in concentrations which did not discernably affect the chick embryo fragments. Kuwata and Shiba (1955) found that glucose was essential for replication of virus in the same system, although no difference in the utilization of this carbohydrate by infected and uninfected cultures was seen.

This literature survey of the metabolism of tissue fragments infected with various viruses revealed that study of the amount of virus produced by these tissues under various conditions permitted a true beginning of the interpretation in biochemical terms of the cell-virus complex. Concepts of energy requirements for viral replication as well as ideas of altered metabolic pathways in virus infected cells emerged. In addition, the necessity for specific amino acids, purines, pyrimidines, and vitamins in production of virus by certain tissues was established.

Employment of tissue fragments in metabolic studies of viral infection at the cellular level possesses intrinsic disadvantages. An ideal system should be composed of one

cell type only, and the cells at the time of infection should be all in the same physiological state. These requirements are necessary so that any observed differences between infected and non-infected populations may be attributed to the infection, and not to pre-existing differences between the cell populations. A tissue fragment obviously consists of a variety of cell types and the ages and physiological conditions of the individual cells vary greatly. Furthermore, in an ideal system the proportion of cells infected, as well as the time of infection, should be known accurately. Only under such circumstances may changes due to infection be followed with respect to time. With a tissue fragment it is impossible to ascertain exactly the number of infected cells or the time that individual cells are infected. Moreover, the physical and chemical environment of all cells in an ideal system should be uniform. Only under such circumstances may differences between infected and normal cells be attributed with certainty to the infection. Nutrients of the medium and oxygen are not as readily available to a cell deeply situated within a tissue fragment as to a cell located near the surface. The crowding together of respiring cells produces localized gradients of decreased oxygen tension, increased carbon dioxide, decreased pH, increased

ammonia tension, decreased concentration of nutrients and vitamins, and increased temperature. Therefore, the value of tissue fragments in quantitative studies of the metabolism of infected cells is severely limited.

Metabolism of Cell Cultures Infected by Virus

The inadequacies of cultured tissue fragments for study of the biochemistry of viral infection at the cellular level may be avoided to a large extent by the employment of established cell cultures. These are homogeneous in cell type and reasonably so in the physiological state and age of the population at the time of exposure to virus. All cells of such cultures may be exposed to an accurately determined number of infectious particles at a precisely determined time, and the proportion of cells which become infected may be discerned (Stoker, 1959). The physical and chemical environment of the individual cells in a monolayer is uniform, definable, and controllable within narrow limits. These advantages of cell cultures over tissue fragments have led to extensive use of the former in the exploration of virus-host relationship.

Alterations of Cellular Constituents and Products by Viral Infection

The most direct approach to the elucidation of biochemical events in the course of viral infection consists of chemical analyses of the changes in constituents and products of the infected cells with respect to time.

Metabolism of nucleic acid. Because nucleic acids are prominent in the composition of viruses, the effects of infection on nucleic acid metabolism have been studied most extensively. Levy et al. (1957) investigated the nucleic acid metabolism of HeLa cells infected with adenovirus type 2. Such cells exhibited a definite increase in uptake of labeled phosphorus in comparison with normal cells. This stimulation was expressed before infectious virus was produced. No increase was detected in cellular nucleic acid as a result of infection.

Cooper (1957) compared the paths of phosphate transfer in normal chick embryo cell monolayers and those infected with vesicular stomatitis virus. There was no detectable effect of infection on the rate of phosphorus uptake by nucleic acid or by other phosphate containing fractions until about half-way through the exponential release period. At this time, phosphorus uptake became reduced drastically.

A great decrease in sucrose-soluble ribonucleic acid was noted late in the infection. These changes may have been secondary effects of infection, rather than primary biochemical lesions caused by the virus.

Rosenbergova (1960) described an earlier effect of infection with Newcastle disease virus on chick fibroblasts. In comparison with normal cells, a significant increase in nucleic acid metabolism occurred during the first three hours after exposure to virus.

Joklik (1959) and Joklik and Rodrick (1959) investigated changes in nucleic acid of cultures of several cell strains after infection with vaccinia virus. Infection increased the rate of incorporation of labeled adenine into microsomal ribonucleic acid, although ribonucleic acid is not an integral part of vaccinia virus. Joklik postulated that the stimulation of ribonucleic acid metabolism was connected with modification of the protein synthesizing mechanisms of the cell that was necessary for the formation of viral protein. Ackermann and Loh (1960) found that within 24 hours after exposure of HeLa cells to vaccinia virus there occurred an increase of more than 100 percent in cytoplasmic ribonucleic acid, whereas no increase in ribonucleic acid was noted in normal cell cultures. It is relevant that

the increase began as early as four hours after infection although intracellular virus was first detected eight hours after infection. Deoxyribonucleic acid synthesis was stimulated by infection also.

Nucleic acid metabolism of HeLa cells infected with poliovirus has been investigated most extensively. Miroff et al. (1957) noted that a large incorporation of labeled phosphorus into the virus infected cells occurred in the presence of a medium incapable of supporting cellular growth. These workers ascribed the uptake either to synthesis of virus or to an attempt by the cells to maintain themselves in the presence of virus. Massab et al. (1957) found that cytoplasmic ribonucleic acid of infected HeLa cells increased 250 percent over that of normal controls, and that virus increased concomitantly. However, Ackermann et al. (1959) concluded that most of the newly formed nucleic acid was not virus. This interpretation arose from observations that the base ratio of the newly formed nucleic acid was characteristic of the cell and not of the virus, that the distribution of the nucleic acid in the cell fractions did not correspond to the distribution of infectious virus and that the increase in nucleic acid was far in excess of the amount of virus produced. Goldfine et al. (1958) reported that the incorporation

of cytidine into ribonucleic acid of HeLa cells infected by poliovirus continued at nearly normal rates, whereas uptake of the nucleoside by cellular deoxyribonucleic acid decreased. These investigators therefore suggested that poliovirus inhibits deoxyribonucleic acid synthesis directly, while viral ribonucleic acid continues to be synthesized.

Salzman et al. (1959) questioned the suitability of using incorporation of purine and pyrimidines into nucleic acids as a measure of synthesis. The criticism arose from demonstration of incorporation of cytidine into HeLa cell ribonucleic acid in the absence of net ribonucleic acid synthesis. In the same communication Salzman and co-workers reported an early inhibition by poliovirus infection of synthesis of all types of nucleic acid in HeLa cells; these data are in sharp disagreement with those of Ackermann et al. (1959).

Kaplan and Ben-Porat (1959) suggested that changes in nucleic acid metabolism of infected cells may not be related directly to viral synthesis. This suggestion was prompted by the observation that although the deoxyribonucleic acid content of rabbit kidney cell cultures infected with pseudorabies virus increased in comparison to that of uninfected cells, this increase was preventable by X-ray

irradiation at levels which had no effect on production of virus.

Metabolism other than nucleic acid. Studies of changes in normal cellular constituents and products resulting from viral infection of cell cultures have included search for alterations in enzymatic activity. Kovacs (1956b) reported that such alterations occurred in monkey kidney epithelial cell cultures after infection with poliovirus. Activity of several enzymes decreased 24 to 48 hours before viral cytopathogenicity appeared. He proposed that such decreases could be utilized as a quantitative measure of cytopathology. Kaufman and Hill (1960) described a decrease in succinic dehydrogenase activity of HeLa cells as early as six hours after infection with Newcastle disease virus.

Alterations in carbohydrate metabolism of cell cultures by viral infection have been noted also. Levy and Baron (1956) found that infection of monkey kidney cell cultures with poliovirus stimulated both aerobic and anaerobic glycolysis. In agreement, Becher et al. (1958) demonstrated that cell cultures of human amnion consumed more glucose as a result of infection by this virus. Conversely, Gifford and Syverton (1957) reported that infection of HeLa cells with poliovirus did not significantly alter

glycolysis or oxygen consumption of the cells. A greater increase of organic acids in cultures of HeLa cells infected with adenovirus type 4 than in uninfected cultures was reported by Fisher and Ginsberg (1957). Levy et al. (1957) stated that infection of these cells with adenovirus type 2 also increased glycolysis. Green et al. (1958) noted no significant changes in oxygen consumption of human leukemic cells after infection with Newcastle disease or mumps viruses. However, the cultures which were infected with Newcastle disease virus exhibited a marked increase in aerobic glycolysis. Both glucose-6-phosphate dehydrogenase and hexokinase activity of rabbit kidney cell cultures increased following infection by herpes simplex virus (Scott et al., 1961).

Obviously, some insight into the chemical events leading to viral replication has been gained by analysis of chemical changes in cells with respect to time after infection. That more information has not been obtained is attributable largely to limitations intrinsic to this approach. For example, the infection with virus may bring about a diversion of host energy and cellular products in amounts so small as to be unrecognizable by the sensitivity of present methods. Nevertheless, this diversion may have disastrous consequences for the host cell provided it occurs

at a critical point in the cellular economy.

Nutritional Requirements of Cells

for Production of Virus

Detailed information of the reactions upon which viral replication depends may be obtained by an approach other than analysis of cellular changes relative to time, namely by study of the effects of environment on the biology of viral reproduction. The environment in which a virus must multiply is the interior of a cell. The intracellular milieu is dependent largely upon the external environment, which is amenable to precise definition and control in the case of cell cultures. Therefore, the nutritional requirements for viral replication may be established and it is possible that understanding of the reactions by which the cell produces virus may be gained. Variation of cellular environment has been achieved either by the employment of enzyme inhibitors and metabolite analogues, or by quantitative and qualitative alteration of the cell culture medium.

Enzyme inhibitors and metabolite analogues. The dependence of a cell upon a particular enzymatic reaction for production of virus may be established by demonstrating that suppression of viral synthesis occurs in the presence of a

specific inhibitor of the reaction. The concentrations of the inhibitor employed must not affect the cell directly. Becher et al. (1958) discovered that certain concentrations of potassium cyanide and sodium azide stimulated glucose uptake both by human amnion and by monkey kidney cell cultures without influencing either cell viability or poliovirus synthesis. The interpretation offered was that the inhibitors induced an increase in the rate of glycolysis by blockage of the tricarboxylic acid cycle. Since inhibition of the oxidative reactions was not inimical to viral multiplication, energy for viral replication appeared to be derived from glycolysis. Polatnick and Bachrach (1960) reached the same conclusion from a study of bovine kidney cell cultures infected with foot-and-mouth disease virus, since production of virus was affected more by poisons of the glycolytic cycle than by those inhibitory to the citric acid cycle. Franklin (1958) used concentrations of proflavin which were capable of selective inhibition of cytoplasmic protein formation in order to establish that the S antigen of influenza virus is produced in the cytoplasm. The investigations regarding enzyme inhibitors can be summarized by stating that indications of the importance of various metabolic pathways to viral replication have been

obtained in certain instances.

Similarly, the employment of metabolite analogues has been a valuable research tool. Inhibition of viral production by a concentration of an analogue which is not directly viricidal, which does not prevent infection of the cell, and which does not demonstrably affect cellular metabolism, is highly suggestive that the corresponding metabolite is directly involved in synthesis of virus. In such a manner, Ackermann et al. (1954) demonstrated the necessity of phenylalanine for poliovirus multiplication in HeLa cell cultures by use of fluorophenylalanine. The analogue had no effect on viral yield if added four or more hours after infection. From this observation, the investigators concluded that phenylalanine was required only in the early phase of replication of virus. Loh (1960) observed that an antagonist of folic acid blocked synthesis of vaccinia and herpes simplex viruses by mammalian cell cultures. Production of influenza virus was unaffected. The concentration of the analogue which was employed blocked production of cellular deoxyribonucleic but not ribonucleic acid. It is perhaps pertinent that influenza virus is the only one of the three viruses mentioned which contains ribonucleic acid rather than deoxyribonucleic acid. Pollard et al. (1960) described the

combined use of cytochemical and metabolite analogue techniques for elucidation of the developmental stages of psittacosis virus in human synovial cell cultures. The host cell contributed a ribonucleic acid matrix around the infecting deoxyribonucleic acid viral particle and soon other deoxyribonucleic acid viral particles appeared within the matrix. However, in the presence of 5-fluorouracil, the matrix developed abnormally and no infectious virus was formed. Therefore, cellular ribonucleic acid seemed to be involved in production of viral deoxyribonucleic acid. Manson et al. (1960) demonstrated that poliovirus production by HeLa cells in the presence of deuterium oxide was increased 20 percent over production by control cultures. The investigators suggested that this effect might be due to a prolonged eclipse period in the presence of the deuterium oxide.

In order to obtain valid information of the chemistry of viral replication, the analogues selected must interfere with synthesis of virus per se, rather than with essential metabolism of the host cell. Gifford et al. (1954) indicated that this stipulation may not always have been met. These workers demonstrated that several analogues suppressed viral multiplication only when used in concentrations inhibitory

to cellular respiration. Worthy of note is that such concentrations gave no cytological evidence of damage.

Thiosemicarbazones are reported to have some inhibitory activity against poliovirus in mice (Hamre et al., 1951; Bauer, 1955). Sheffield et al. (1960) showed that isatin thiosemicarbazone also protects cultures of embryo rabbit kidney cells from vaccinia and rabbitpox viruses. Since the concentration employed neither inactivated the viruses in vitro nor affected adsorption, the inference was drawn that protection resulted by interference with some intracellular process.

Nutritionally deficient media. The importance of a specific nutrient in the production of a virus by a certain cell may be assessed by examination of the effect on viral replication of omission of that nutrient from the cell culture medium. Once the requirement for a nutrient is established, efforts may be directed toward identification of the essential chemical reaction in which it is involved in viral multiplication.

Dulbecco and Vogt (1954) were able to demonstrate that production of Western equine encephalomyelitis virus by chick embryo cell cultures in a medium of serum in balanced salt solution was increased five-fold by addition of

embryo extract to the medium. The constituents of the extract which were responsible for the enhancement were unknown. Morgan and Bader (1957) produced a latent infection in cell cultures of mouse fibroblasts with psittacosis virus by starving the cells on a balanced salt solution before exposure to virus. The infection became overt upon addition of a complex medium. As in the work of Dulbecco and Vogt (1954), identification of the nutrients responsible for the production of virus was not accomplished.

It was apparent that precise identification of the nutritional requirements for proliferation of virus would be facilitated greatly by developing a completely defined medium for the cells employed. Eagle (1955a) contributed much in this direction by establishing the nutritional needs of HeLa cells in tissue culture. He learned that HeLa cells required 13 amino acids, eight vitamins and a carbohydrate for growth in a balanced salt solution which contained dialyzed serum. This information made possible the study of the virus-cell relationship under well defined and closely controlled conditions. The composition of the synthetic medium can be varied at will and the effect of such variation on the extent of viral propagation can be measured. From such studies, the nutritional factors crucial for synthesis

of virus may eventually be determined.

Eagle and Habel (1956) found that HeLa cells produced very little virus when glutamine was omitted from otherwise complete Eagle's medium. On the other hand, the cells produced almost as much virus in a simple salt solution as in the complete medium if glutamine and glucose were added to the salt solution. These observations were interpreted as indicating that the role of glutamine in viral replication was more than merely a preservation of the integrity of the metabolic processes necessary for viral synthesis. A direct participation of this compound in viral multiplication was suggested. It was postulated that one function of glutamine was to furnish nitrogen for the synthesis of viral nucleic acid. Further investigation (Darnell and Eagle, 1958) revealed that glutamic acid at higher concentrations satisfied the glutamine requirement.

The demonstration by Eagle and co-workers of the importance of glutamine in production of poliovirus is only one of many indications of the fundamental part played by this compound in cellular reactions. Fischer (1948) showed that glutamine greatly improved the growth of chick embryo myoblasts and osteoblasts in vitro. Ehrensverd et al. (1949) noted that the maintenance of embryo chicken heart fibroblast

cultures required the presence of this compound. A thorough investigation of the glutamine requirement of HeLa cells was conducted by Eagle et al. (1956), who found that the cells died within a few days when supplied with Eagle's basal medium minus glutamine containing five percent dialyzed human serum. D-glutamine could not substitute for L-glutamine. The optimal concentration was two millimoles, and no growth occurred at concentrations below 0.5 millimole. Glutamic acid at higher concentrations replaced glutamine for HeLa cells, but not for a cloned strain of mouse fibroblasts. Neuman and McCoy (1956) showed that glutamine was vital for in vitro cultivation of Walker carcinosarcoma 256. Ehrlich ascites carcinoma cultures failed to survive in the absence of glutamine (Rabinovitz et al., 1956), and the same was true for uterine fibroblast cultures (Swim and Parker, 1958). The compound is utilized extensively by cell cultures of human foetal lung, kidney and liver (Sinclair and Leslie, 1959). In fact, glutamine has been found to be essential for the survival of every cell culture in which the requirement has been investigated, with the exception of chick heart fibroblasts (Morgan and Morton, 1957). The only disagreement with this generalization appears in a communication by Pasieka et al. (1958), in

which glutamine was said to be a non-essential growth factor for a cloned strain of mouse fibroblasts in completely synthetic medium although playing a major role in regulation of amino acid metabolism.

The nature of the key role of glutamine has been sought by several workers. Eagle et al. (1956) showed that the failure of glutamic acid to substitute fully for the glutamine requirement of HeLa cells was not due to differential permeability. Levinton et al. demonstrated in 1957 that glutamine carbon was incorporated into the glutamic acid, aspartic acid, and proline of HeLa cells. However, glutamine was not the sole source of these three amino acids. Glutamine carbon was utilized to a limited degree in synthesis of alanine, serine, and glycine. The amide nitrogen of glutamine, however, did not contribute to the alpha-amino groups of HeLa cell protein, but did appear as asparagine amide nitrogen. This nitrogen transfer to asparagine was proven by Levinton (1957) not to pass through the free ammonia pool of the cell. Salzman et al. (1958) found that glutamine carbon was incorporated into purine of HeLa cells to some extent, although utilization in cellular pyrimidine was ten-fold greater. Glutamine nitrogen also appeared in pyrimidine bases, and the metabolic

sequence proposed for this conversion was incorporation into pyrimidine of aspartic acid nitrogen which arose from glutamine. In summary, glutamine is known to be a key metabolite in the nutrition of mammalian cells; it enters into such diverse reactions as amide exchange (Waelsch, 1952), transamination (Braunstein, 1947), purine biosynthesis (Goldthwait et al., 1954; Hartman et al., 1955), and is, of course, directly incorporated into protein. In fact, glutamine is likely to be the limiting amino acid involved in protein synthesis (Kvamme and Svenneby, 1961).

A carbohydrate source, as well as glutamine, is crucial to the metabolism of cell cultures. Carbohydrate is important both as a source of energy and of intermediates which may serve as precursors in the viral synthetic process (Polatnick and Bachrach, 1960). In view of the importance of carbohydrates in cellular reactions, it is surprising that a review of the literature reveals only one instance in which the role of this class of compounds in viral production by cell cultures had been investigated extensively by the use of nutritionally deficient medium. Darnell and Eagle (1958) observed that fructose at high concentrations would substitute for glucose in the synthesis of poliovirus by HeLa cells. Several other carbohydrates

supported only slight production of virus at concentrations in excess of those which permitted the growth of HeLa cells in glucose-free medium. A simple balanced salt solution which contained glucose and glutamine supported production of substantial amounts of virus. With increasing concentrations of glucose in the salt solution, the average viral yield per cell began to rise at about 0.2 millimole and maximum production was reached at about five millimoles with the final viral titer at 20 to 30 percent less than when glutamine was included.

A study of the roles of glutamine and glucose in production of virus by cell culture is the subject of the present investigation. These compounds were selected because of their essential role in cellular metabolism, as well as the relatively advanced state of knowledge concerning their intermediary reactions. It was of interest to determine whether the requirements of HeLa cells for these two compounds in the synthesis of poliovirus (Eagle and Habel, 1956) were peculiar to that particular cell-virus system. Therefore, the influence of glutamine and glucose on production of herpes simplex virus by HeLa cells was investigated.

CHAPTER II

MATERIALS AND METHODS

Virus. The virus used throughout this investigation was the HF strain of herpes simplex virus which was obtained from the Communicable Disease Center, Montgomery, Alabama, where it had been maintained in HeLa cells. Upon arrival, identity of the virus as that of herpes simplex was verified by neutralization and complement fixation tests with known specific antisera.

The virus was propagated through six consecutive cultures of HeLa cells in this laboratory, following which a viral pool was made for infection of cells during this study. Initially, it was considered desirable that this pool contain a minimal amount of nutrients that might be utilized by HeLa cells. Inoculation of cell monolayers from such a pool would contribute negligible amounts of undefined nutrients to media under test for effect upon viral proliferation. Accordingly, efforts were directed toward preparation of a pool of virus by propagation upon HeLa cells

maintained in the balanced salt solution of Earle (EBSS) (1943) from which glucose had been omitted.

In the first attempt to prepare such a viral pool the growth medium (GM) of a cell monolayer was removed and the monolayer was washed three times with EBSS. Twelve ml of EBSS were placed on the washed monolayer, and the viral inoculum was added immediately. This viral inoculum proved to be sufficient at a 1:600 dilution to infect 50 percent of HeLa cell monolayers (6×10^2 TCID₅₀). One TCID₅₀ (tissue culture infectious doses₅₀) is defined as the amount of virus sufficient to infect half of the cultures of a specified system under defined conditions. The EBSS from the culture contained no demonstrable virus 48 hours following viral inoculation. A second monolayer was inoculated in the same manner with 10^4 TCID₅₀ of virus after replacement of growth medium with EBSS which was deficient in glucose. It was hoped that the increase in size of inoculum might stimulate viral production. Microscopic examination of this monolayer revealed that approximately 80 percent of the cells were dead 96 hours after inoculation. Titration of the EBSS at this time showed a content of $10^{1.5}$ TCID₅₀ of virus per ml. In the next attempt, the GM was not replaced with glucose-free EBSS until 48 hours after

the cells had been exposed to virus. It was hoped that this delay would allow cellular virus to develop sufficiently before the replacement in order that an adequate quantity of infectious units would be synthesized by the cells in the presence of the salt solution only. However, cellular destruction was so extensive before removal of GM that little virus was produced.

In the final attempt to obtain a nutrient-free viral pool, 2.5×10^5 TCID₅₀ of virus were added to each of two monolayers of HeLa cells, and the interval between infection and replacement of GM with glucose-free EBSS was decreased to 24 hours. The goal in use of this interval was to prevent viral destruction of cells and to enable the cells to complete synthesis of virus in the salt solution. The EBSS removed from the monolayer 24 hours after it had been added was titrated for viral content. The amount of endogenous nutrients which might be liberated into the medium by cellular necrosis was expected to be decreased by this period when the cells were in the salt solution. Again the amount of infectious virus, as shown by titration, proved to be too small to be useful.

An evaluation of the importance of a relatively nutrient-free viral pool to the proposed experiments was made

at this time. As a result of this reappraisal a viral pool was prepared by infection of monolayers of HeLa cells maintained in GM. Viral cytopathogenic effect was well advanced three days after infection when the medium was removed from the cellular monolayers and pooled. After the pool was centrifuged lightly to remove cellular debris, the supernatant fluid was distributed in 0.5 ml amounts in glass ampules. The ampules were sealed by means of an oxygen flame and stored immediately at -65° C until used. The titer of virus sampled from randomly selected vials was found to be $10^{8.2}$ TCID₅₀ per ml. The pool proved to be free of bacterial and fungal contamination.

Antisera. A pool of human sera was used for neutralization of herpes simplex virus in certain experiments to be described. The pool was sterilized by Seitz filtration and stored at -65° C until used. In the neutralization tests to be described, 0.5 ml of a 1:256 dilution of the serum protected 50 percent of the chick embryos against 2.3 times the amount of virus which infected 50 percent of the embryos in the absence of serum.

Cells. HeLa cells were employed in this investigation. This line of human epidermoid cells was cultivated originally by Gey et al. (1952) from a cervical carcinoma. The strain

used in the present study had been propagated serially 40 times in this laboratory prior to the beginning of this investigation.

The cells were propagated routinely in stationary bottles of 250 ml capacity placed in a horizontal position. When a confluent layer of cells had developed on the surface of a bottle, the 12 ml of GM were replaced with 10 ml of 0.2 percent trypsin solution. The culture was incubated for six minutes at 37° C, during which time the cells became detached from the glass and essentially monodispersed. The cells were sedimented immediately by ten minute centrifugation in 10 ml centrifuge tubes with a Model CL International Clinical Centrifuge. The supernatant fluid was discarded and the pellet of cells was resuspended in GM. The concentration of cells in this suspension was determined by hemocytometer count (Melnick, 1956), and the suspension was diluted with GM to approximately 200,000 cells per ml. Sub-cultures of cells were made by inoculation of 250 ml bottles with this suspension. Each bottle received 12 ml of the suspension. A uniform suspension of the cells was maintained by agitation of the medium with a magnetic stirrer during the distribution of cells. One bottle usually provided sufficient cells for three others. The bottles were

closed by means of screw caps. The carbon dioxide which each culture produced was sufficient for growth of the cells. The incubation temperature was 37° C. The medium was renewed every third day. Confluent monolayers of cells usually developed within four days.

Media and solutions. The basal medium developed by Eagle (1955a) was used for propagation of HeLa cells. This medium consisted of EBSS containing 13 amino acids and eight vitamins in the concentrations shown in Table 1. A carbohydrate source was provided by the 0.1 percent glucose of the EBSS. Eagle (1955a) showed that addition of serum to this medium is necessary for the proliferation of HeLa cells. The commercial Eagle's basal medium supplied by Microbiological Associates, Bethesda, Maryland was complete as supplied except for serum and glutamine. GM for the present investigation consisted of this commercial medium to which ten percent calf serum and 2 millimoles of glutamine had been added.

In certain experiments in which constituents other than glutamine and serum were modified, it was necessary to make the medium rather than to obtain it commercially. Concentrated stock solutions of the components were prepared and stored as recommended by Eagle (1955a), and the

TABLE 1

AMINO ACIDS AND VITAMINS OF EAGLE'S BASAL MEDIUM

<u>L amino acids</u>	<u>mM</u>	<u>Vitamins</u>	<u>gm/ml</u>
Arginine	0.1	Biotin	10^{-6}
Cystine	0.05	Choline	10^{-6}
Glutamine	2.0	Folic acid	10^{-6}
Histidine	0.05	Nicotinamide	10^{-6}
Isoleucine	0.2	Pantothenic acid	10^{-6}
Leucine	0.2	Pyridoxal	10^{-6}
Lysine	0.2	Thiamine	10^{-6}
Methionine	0.05	Riboflavin	10^{-7}
Phenylalanine	0.1		
Threonine	0.2		
Tryptophane	0.02		
Tyrosine	0.1		
Valine	0.2		

medium was compounded according to his description. Freshly prepared media were used in each experiment.

When media of identical composition except for one or a few components were to be compared in an experiment for effect upon viral proliferation by cells, a single solution was prepared which possessed all components common to every medium of the particular experiment. The completed experimental media were made from portions of this common solution.

Lactalbumin hydrolysate medium, employed in certain experiments, was prepared as suggested by Melnick (1955). The complete medium consisted of 0.5 percent lactalbumin hydrolysate and ten percent calf serum in Hank's balanced salt solution.

Phenol red, at a final concentration of 20 mg per liter, was included in all media in order to follow pH changes during cellular growth. This concentration of phenol red is used routinely in cell culture media (Melnick, 1956). To control bacterial contamination, 100 units of penicillin and 100 micrograms of streptomycin were added to each ml both of the lactalbumin hydrolysate medium and of Eagle's basal medium. All media were sterilized by Seitz filtration.

EBSS, prepared according to the method of Earle (1943), was employed for washing of cellular monolayers. Hank's balanced salt solution was made by the technique of Melnick (1955). The trypsin solution used in transfer of cell cultures consisted of a 0.2 percent solution of 1:250 trypsin in phosphate-buffered saline which lacked magnesium and calcium (Melnick, 1955). All solutions were sterilized by Seitz filtration.

The bovine serum component of the cell culture media was derived from blood collected from calves approximately one year of age at a local abattoir. Fresh blood was allowed to clot at room temperature and the clot was ringed. The serum was removed after the clotted blood had been left at 4° C overnight for retraction of the clot. Centrifugation of the serum at 5000 x g for 11 minutes removed the remaining red blood cells. A pool of serum from several calves was sterilized by Seitz filtration, distributed in 40 ml volumes in glass vials and stored at -20° C until used. Sufficient serum was contained in two pools to complete the investigation.

Samples. The volumes of samples removed from monolayer bottles were restored by an equal quantity of EBSS at the time of sampling so that the volumes of media

remained constant throughout an experiment. Samples were frozen quickly after removal from the experimental bottles and stored at -65°C in tightly stoppered test tubes. They were titrated for viral content as soon as cellular monolayers were available, and no sample was held for longer than 21 days before titration.

Titration. Viral concentration of samples was determined routinely by infection of HeLa cell monolayers grown in test tubes. In order to obtain the tubed monolayers, a cellular suspension was prepared as described for transfer of cell cultures in bottles. The suspension of cells in GM for inoculation of tubes contained approximately 300,000 cells per ml. From this cell concentration monolayers were grown within three to five days following cellular transfer. Each tube received 0.5 ml of the cell suspension, after which the tubes were closed with rubber stoppers. Next, the tubes were incubated at 37°C in stationary cell culture racks which held them at a five degree angle from the horizontal. The tubes were used when the monolayers were approximately 90 percent confluent. At this time, the cellular population was still in the phase of logarithmical growth (Pace and Layon, 1960).

Each monolayer was examined microscopically before use and those which were unsatisfactory because of contamination or insufficient cellular growth were discarded. Satisfactory tubes were labeled with sample and dilution designation, and the GM was removed. Next, GM was measured in 0.9 ml amounts into tubes to be used for preparation of the sample dilutions. At this time each sample to be tested for viral content was removed from -65° C storage and thawed rapidly in a 37° C water bath. One-tenth ml of this sample was placed in each of the appropriately labeled replicate tubes of monolayers, and 0.1 ml was added to a dilution tube also. This 1:10 dilution was mixed ten times by taking up and releasing the fluid vigorously with a pipet. The correspondingly labeled monolayer tubes were inoculated, a further 1:10 dilution was made and the procedure was repeated until monolayers had been inoculated with the required dilutions of virus. A fresh pipet was used for each dilution. Following inoculation, all tubes were replaced in the stationary racks and incubated for 90 minutes at 35° C, in order to allow maximal adsorption of virus to cell before thermal inactivation occurred (Stoker, 1959). After the adsorption period, 0.4 ml of GM was added to each tube, and the tubes were reincubated at 37° C in the

stationary racks. The medium was renewed on the third day after inoculation.

Most experiments included comparison of viral production by monolayers nourished by media which differed in composition. All samples obtained from these monolayers at the same interval after infection were titrated for viral content on the same day. Tubed monolayers prepared from the same cellular suspension were used in these comparative titrations. Titration tubes were randomized before inoculation with the samples so that results would not reflect a possible variation in condition of the different monolayers. Four tubes for each dilution of sample were employed routinely in the experiments. Monolayers were examined microscopically at magnifications of 35 and 100 for viral cytopathogenic effect at two, five, and seven days after inoculation.

Certain viral titrations are described later as having been performed with embryonated eggs. Twelve-day-old eggs from white Leghorn hens were supplied by a local hatchery. Inoculation was performed by a modification of the method of Beveridge and Burnet (1946). An egg was candled and a location on the shell over a relatively avascular portion of the underlying chorioallantoic membrane (CAM)

was marked. This area of the shell, as well as that over the air sac, was cleansed with 70 percent alcohol. A small hole was made through the shell with a sterile metal egg punch at the mark over the CAM, and another hole punctured at the air sac end of the shell. Care was taken not to penetrate the egg shell membrane during this operation. With the egg placed horizontally in a suitable holder, a drop of sterile gelatin saline was placed on the hole above the CAM in order to reduce trauma upon formation of the false air sac. A 24-gauge needle, bevel up and almost parallel to the shell, was inserted through the saline in such a way as to pierce the shell membrane. Upon withdrawal of the needle, the gelatin saline solution slowly seeped into the egg between the shell membrane and the underlying CAM. Gentle suction was applied to the terminal hole over the air sac while the membrane was observed with the use of the candler. Entrance of air through the hole in the shell over the CAM caused a "false" air sac to be formed under this hole, as indicated by a disappearance from view of the clearly discernible blood vessels.

Serial dilutions of the sample to be tested for viral content were prepared as previously described for titration of tubed monolayer samples. One-tenth ml of inoculum was

injected into the false air sac by means of a tuberculin syringe fitted with a 27-gauge, $\frac{1}{4}$ th inch needle. The egg was rocked gently immediately following inoculation in order to distribute the inoculum over the CAM of the false air sac. After the egg was reincubated at 37° C for 72 hours in a horizontal position with the false air sac uppermost, the CAM was examined for presence of viral lesions. In this procedure, the part of the CAM directly below the false air sac was removed by cutting with scissors. This portion of CAM was spread in a Petri dish. The titer of a sample was determined by the number of viral specific lesions on membranes which resulted from inoculation with dilutions of the sample, since each lesion arises from one infectious unit in a properly diluted inoculum (Scott, 1956). A minimum of four eggs was inoculated with each dilution of the samples that were titrated in eggs.

Neutralization test. The concentration of specific neutralizing antibody against herpes simplex virus in the pool of human sera was determined as recommended by Scott (1956). The serum was inactivated at 56° C for 30 minutes and two-fold dilutions were mixed with a viral suspension. The infectivity of the suspension of virus was determined by previous titration. The mixtures were allowed to incubate

for one hour at 37° C, and then were inoculated into the yolk sac of seven day old chick embryos. Each embryo received 0.5 ml of inoculum. The embryos were incubated at 37° C and candled twice daily. Those deaths occurring before 48 hours post-inoculation were considered to be non-specific. The herpes simplex virus neutralizing antibody titer of the pooled human sera was indicated by the greatest dilution of serum which resulted in protection of 50 percent of the chick embryos against the challenge virus.

CHAPTER III

RESULTS

The minimal amount of calf serum to be employed in Eagle's basal medium (EBM) was determined. Modifications of this medium were used in definition of specific nutritional requirements of HeLa cells for production of herpes simplex virus. It was necessary, therefore, that media of well-defined composition be utilized, and that the amount of serum, the only undefined component of EBM, be minimal. Nevertheless, inclusion of serum in the media was desirable, since EBM without serum fails to support proliferation of HeLa cells (Eagle, 1955a). It was essential for certain experiments that the limiting factor in synthesis of virus be the lack of the particular nutrient under test, rather than absence of serum. Therefore, the minimal amount of serum capable of adequately maintaining HeLa cells in EBM was determined.

Monolayers were grown in tubes with EBM containing ten percent calf serum (GM). The tubes were then divided

randomly into five groups. The GM was removed, and each group received EBM containing 0, 0.62, 1.25, 2.5, or five percent calf serum. Immediately and at 24 hour intervals thereafter, monolayers of two tubes from each group were exposed to trypsin solution for ten minutes at 37° C. This treatment resulted in a suspension composed almost entirely of single cells. The numbers of cells in these suspensions were determined by hemocytometer count, and the numbers from replicate tubes were averaged. Results of these determinations are shown in Table 2 and in Figure 1.

It may be seen that definite reduction of the number of cells occurred on the fifth day in EBM which lacked serum and that no cells were detected after one additional day. With 0.62 percent serum, a decrease in cell number was seen earlier, although some cells persisted through the seventh day. In 1.25 percent serum no diminution of population was observed until the seventh day, but all cells had disappeared one day later. At concentrations between 1.25 and 2.5 percent serum there was little difference in ability to maintain the cells. However, no decrease occurred through seven days in the number of cells supported by EBM containing five percent serum, and cells were present at the conclusion of the experiment on the eighth day.

TABLE 2

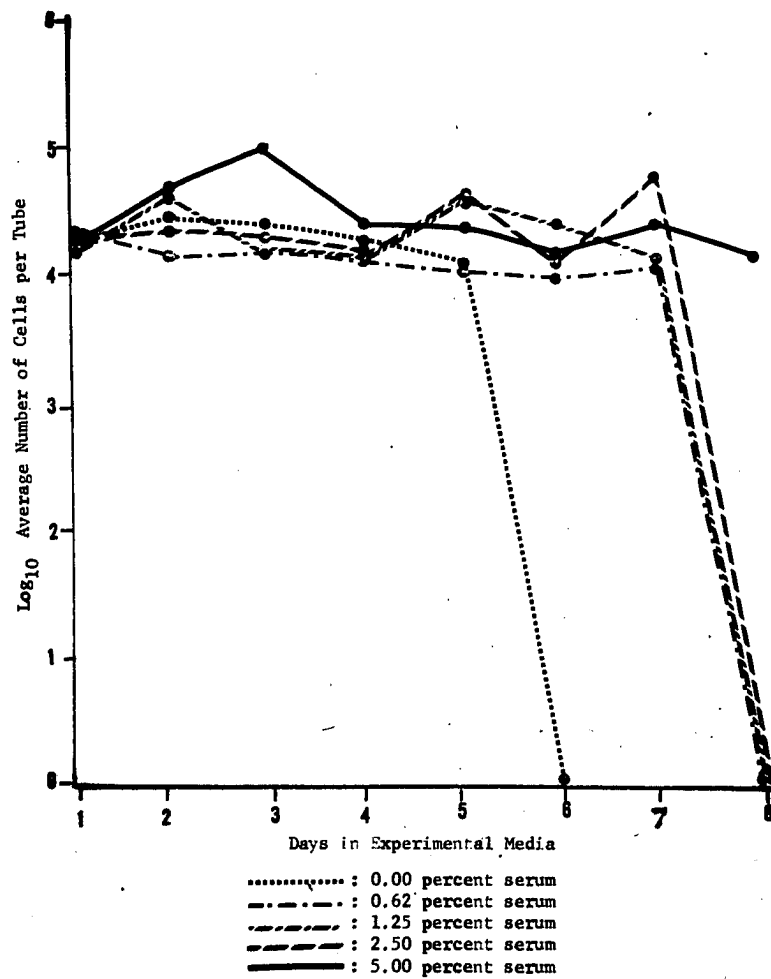
SURVIVAL OF HELA CELLS IN EAGLE'S BASAL MEDIUM CONTAINING
VARIOUS CONCENTRATIONS OF CALF SERUM

Days in Test Media	Number ^a of Surviving Cells per Tube				
	Percent calf serum in medium				
	0	0.62	1.25	2.5	5.0
1	3.0	3.5	1.5	2.5	2.5
2	4.5	1.5	6.0	3.0	6.5
3	3.75	1.5	1.5	3.0	10.5
4	2.25	0.75	1.25	2.25	3.75
5	0.75	0.5	5.5	6.0	3.75
6	0	0.25	3.75	1.2	1.5
7	0	0.75	1.0	9.0	3.75
8	0	0	0	0	1.0

^a Tabular number multiplied by 10^4 equals total number,
i.e., 3.0 = 3.0×10^4 .

FIGURE 1

SURVIVAL OF HELA CELLS IN EAGLE'S BASAL MEDIUM
CONTAINING VARIOUS CONCENTRATIONS OF CALF SERUM



Further evidence for the adequacy of five percent serum for maintenance of the cells was gained from the fact that this was the only concentration tested in which the medium became more acidic with passage of time, an indication of an actively metabolizing monolayer.

At concentrations of serum tested, a few fusiform cells were detected by microscopic examination long after the hemocytometer counts were zero. These few cells clung to the glass surfaces of the tubes for ten days in absence of serum, for 23 days in 0.62 percent serum, for eight days in 1.25 and in 2.5 percent serum, and for 19 days in five percent serum.

Experiments were designed to determine if HeLa cells maintained in EBM deficient both in glucose and glutamine were capable of synthesizing herpes simplex virus. These experiments were planned to demonstrate also the effect on viral production of cellular maintenance on deficient medium for varying lengths of time.

Eagle's basal medium containing five percent serum but no glucose or glutamine was divided into two portions. Glucose and glutamine were added to one of the portions to make it a complete medium. Monolayers of HeLa cells which had been propagated in tubes in commercial GM were washed

twice with glucose-free EBSS in order to remove traces of the medium. The tubed monolayers were divided next into two groups. The complete growth medium was added to the tubes of one group. Immediately and at 12 hour intervals after the addition of test media, the viral inoculum was added to the medium which covered the monolayers in tubes from each group. Each tube inoculated received 100 TCID₅₀ of virus suspended in 0.1 ml of glucose-free EBSS. Every 24 hours after inoculation of a set of tubed monolayers fluid was removed for titration from two of the tubes which contained the deficient medium and from two with complete medium. At the time each sample was taken, the numbers of cells were determined in uninoculated monolayers which had been nourished by the same medium throughout the experiment.

The cytopathogenic effect produced by herpes simplex virus on a HeLa cell monolayer is pictorially shown in Figure 2. The picture of the infected monolayer may be contrasted with that of the uninfected monolayer as shown in Figure 3. It may be seen that use of this titration system allowed the presence of infectious virus in the samples to be recognized clearly. Viral cytopathogenic effect was observed only rarely in one or more tubes of a dilution without also being present in all tubes of the next lower dilution.

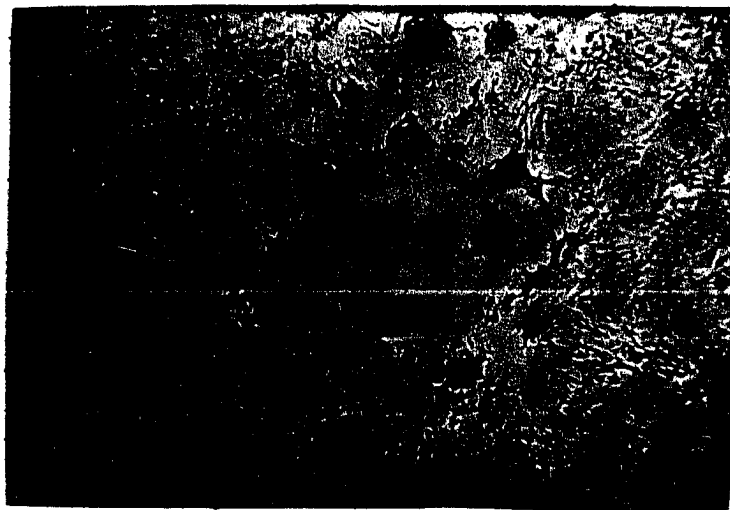


Figure 2. - HeLa cells infected with herpes simplex virus (X 100).



Figure 3. - Normal uninfected HeLa cells (X 100).

Usually all tubes of the lower dilution were infected.

Representative results of the titrations are presented in Table 3 and shown graphically in Figures 4, 5, 6, and 7. That samples taken immediately upon inoculation contained virus in the amounts which had been inoculated indicated that all tubes except the 84 hour series had received the same number of infectious units. While all tubes in the group inoculated 84 hours after the experimental medium was added received equal amounts of virus, these amounts proved to be ten times less than those which had been used with the other groups. Complete medium supported viral replication by the cells in all instances except where inoculation of virus had been delayed until 84 hours after the last change of medium. However, when the virus was inoculated 36 hours or longer after the change of medium synthesis of virus neither occurred as soon after inoculation nor persisted as long as when the inoculation was done earlier than 36 hours. The only exception to this observation occurred in the series of tubes with complete medium to which virus was added 72 hours after the change of medium; in this instance virus was detected at 24 hours and was present for as long as 96 hours after inoculation. However, the amount of virus produced was less

TABLE 3

PRODUCTION OF HERPES SIMPLEX VIRUS BY CELLS IN COMPLETE^a AND DEFICIENT^b MEDIA

Hours on Experimental Media before Viral Inoculation	Medium	Log TCID ₅₀ per Monolayer by Hours after Inoculation					Number ^c of Cells Per Uninoc- ulated Monolayer
		0	24	48	72	96	
0	Complete	2	2	4	2	3	2.5
	Deficient	2	0	0	2	2	3.0
12	Complete	2	2	2	4	3	6.0
	Deficient	2	0	0	0	0	3.0
24	Complete	2	2	2	4	1.5	2.5
	Deficient	2	0	0	0	0	2.0
36	Complete	2	0	2	0	0	4.5
	Deficient	2	0	0	0	0	4.5
48	Complete	2	0	4	4	0	7.5
	Deficient	2	0	0	0	0	0.75

60	Complete	2	0	2	2	0	4.5
	Deficient	2	0	0	0	0	0
72	Complete	2	1	1	1	1	3
	Deficient	2	0	0	0	0	0
84	Complete	1	0	0	0	0	1
	Deficient	1	0	0	0	0	0
96	Complete	- ^d	-	-	-	-	0.75
	Deficient	-	-	-	-	-	0

^a Complete medium; Eagle's basal medium.

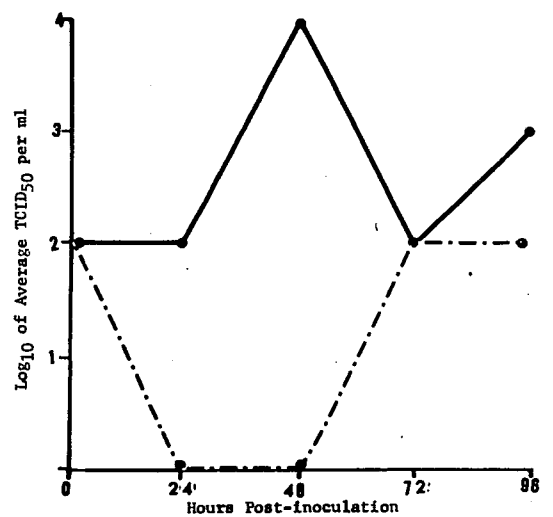
^b Deficient medium; Eagle's basal medium, lacking glucose and glutamine.

^c Tabular number multiplied by 10^4 equals average number of cells per monolayer at time of viral inoculation.

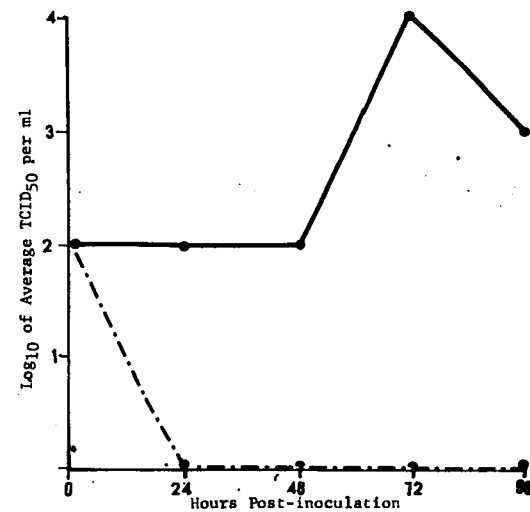
^d -, not done.

FIGURE 4

VIRAL PRODUCTION BY CELLS MAINTAINED WITH COMPLETE^a
OR DEFICIENT^b MEDIA FOR VARIOUS TIMES BEFORE INFECTION



Cells maintained in experimental
media 0 hours pre-inoculation.

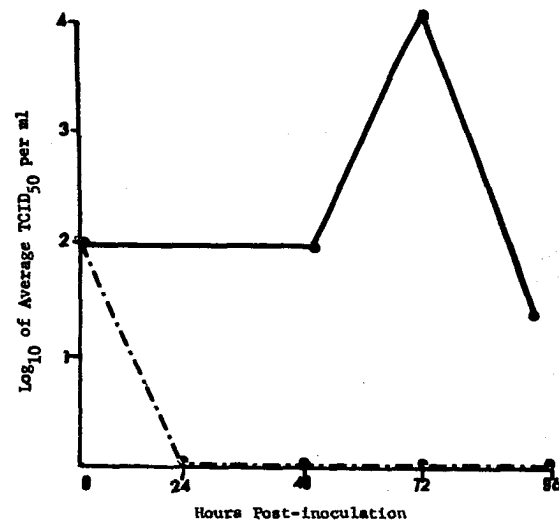


Cells maintained in experimental
media 12 hours pre-inoculation.

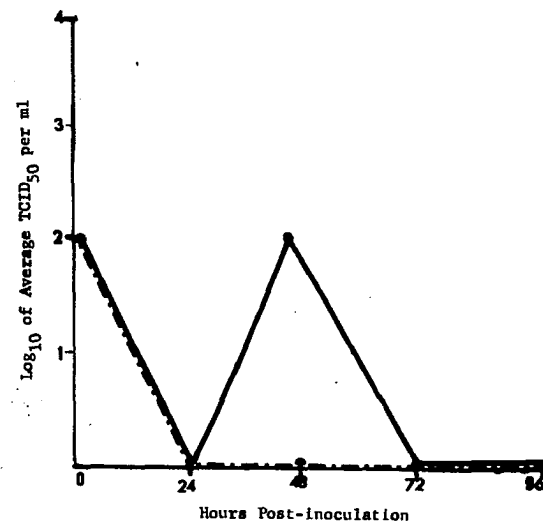
a: Eagle's basal medium.
b: Eagle's basal medium, less glucose and glutamine.
— : Complete medium
- - - : Deficient medium

FIGURE 5

VIRAL PRODUCTION BY CELLS MAINTAINED WITH COMPLETE^a
OR DEFICIENT^b MEDIA FOR VARIOUS TIMES BEFORE INFECTION



Cells maintained in experimental
media 24 hours pre-inoculation.

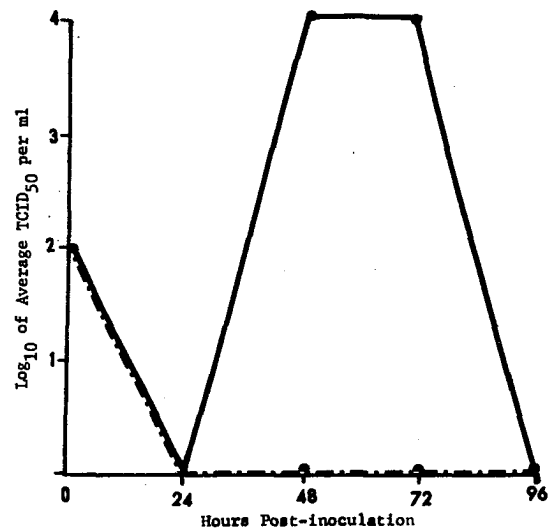


Cells maintained in experimental
media 36 hours pre-inoculation.

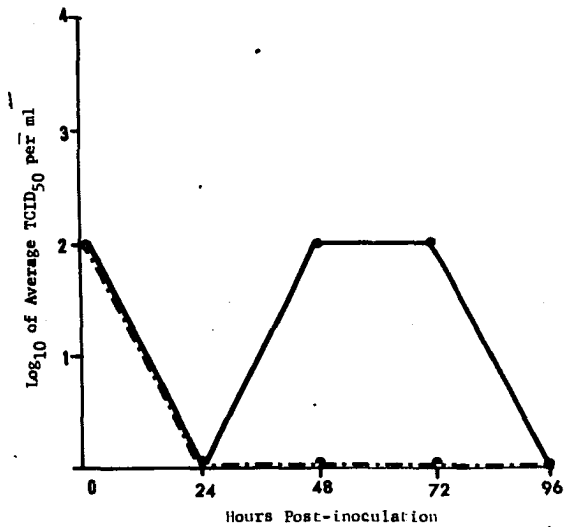
- a: Eagle's basal medium.
b: Eagle's basal medium, less glucose and glutamine.
— : Complete medium
- - - : Deficient medium

FIGURE 6

VIRAL PRODUCTION BY CELLS MAINTAINED WITH COMPLETE^a
OR DEFICIENT^b MEDIA FOR VARIOUS TIMES BEFORE INFECTION



Cells maintained in experimental
media 48 hours pre-inoculation.

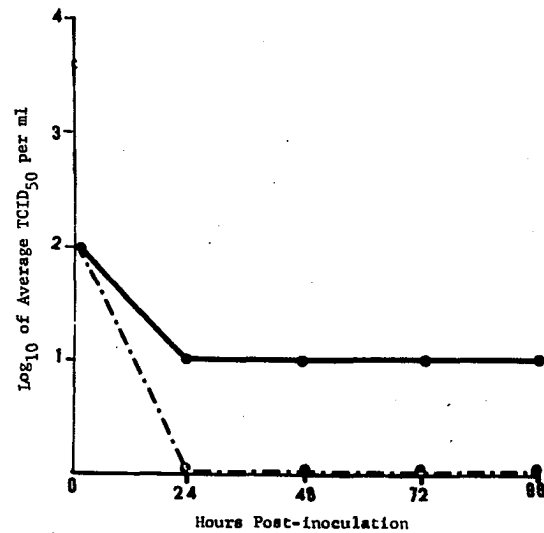


Cells maintained in experimental
media 60 hours pre-inoculation.

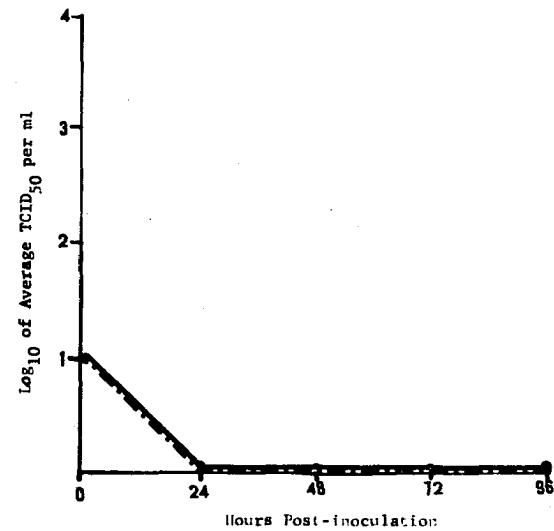
- a: Eagle's basal medium.
- b: Eagle's basal medium, less glucose and glutamine.
- : Complete medium
- - - - -: Deficient medium

FIGURE 7

VIRAL PRODUCTION BY CELLS MAINTAINED WITH COMPLETE^a
OR DEFICIENT^b MEDIA FOR VARIOUS TIMES BEFORE INFECTION



Cells maintained in experimental
media 72 hours pre-inoculation,



Cells maintained in experimental
media 84 hours pre-inoculation.

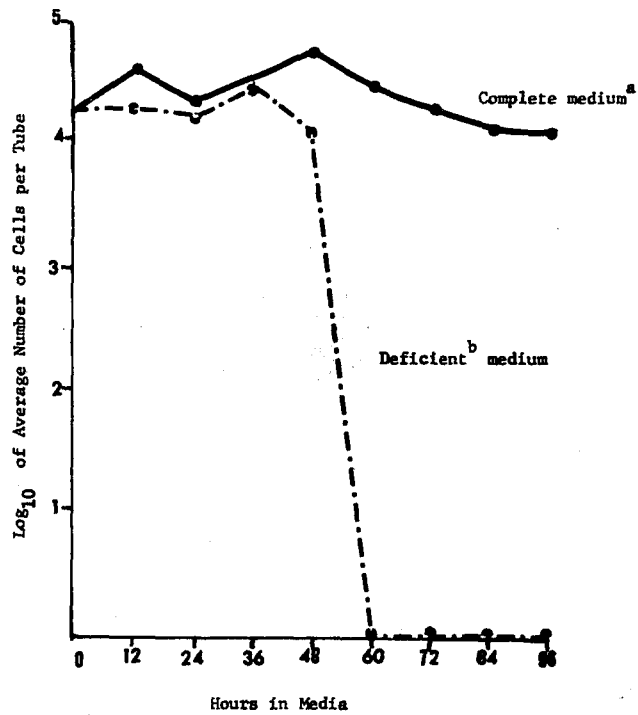
a: Eagle's basal medium.
b: Eagle's basal medium, less glucose and glutamine.
— : Complete medium
- - - : Deficient medium

than that produced when the cells were inoculated within 24 hours of the change of medium. In contrast to the ability of cells in complete medium to support viral synthesis, cells supplied with the deficient medium produced virus only when they were infected immediately after the deficient medium was placed on the monolayers. Even in this instance, viral replication was both delayed and limited in quantity. These results indicate clearly that HeLa cells are incapable of synthesis of herpes simplex virus when supplied with Eagle's basal medium which contains five percent calf serum but which is deficient in glucose and glutamine.

A pertinent question is whether the lack of viral production by cells with deficient medium may be because few cells survive such conditions. Conceivably, as much virus may be formed per cell in the presence of deficient medium as in the presence of complete medium. This question is answered by comparison of averages of cell counts from uninoculated monolayers after various periods in each of the media (Table 3 and Figure 8). Monolayers maintained 48 hours or more in deficient medium decreased markedly in cell population, whereas a comparable decrease in the presence of complete medium was delayed until 84 hours. Conditions of the monolayers, as indicated by hemocytometer

FIGURE 8

SURVIVAL OF HELA CELLS
IN COMPLETE AND DEFICIENT EAGLE'S BASAL MEDIA



a: Eagle's basal medium.

b: Eagle's basal medium, less glucose and glutamine.

counts, were reflected in the microscopic appearance of the cells; morphological changes were obvious 12 hours before decreases in populations occurred. Thus, cells in deficient medium appeared healthy at 24 hours. By 36 hours, many had assumed a fusiform shape, a change which presaged decrease in numbers of living cells at 48 hours. Cells in complete medium became fusiform first at 72 hours. Accordingly, the synthesis of virus by monolayers in complete medium may be compared directly with that by monolayers in deficient medium only during the first 24 hours after addition of the experimental media. Approximately equal numbers of cells were present in each medium during this period, and they were physiologically comparable as judged by morphology. During this period cells in complete medium were capable of synthesis of much more virus than were cells in deficient medium.

The influence of the medium on production of herpes simplex virus by HeLa cells may reflect a lack of viral adsorption to the cells in the presence of deficient medium rather than decreased viral synthesis per se. In order to test this possibility, cells were grown to monolayers with GM in eight ounce glass prescription bottles. On half the monolayers this medium was replaced by the complete

basal medium of Eagle containing five percent calf serum, while the other monolayers received medium that was identical except that it lacked glucose and glutamine. Two hundred TCID₅₀ of virus were added to the six ml of medium in each bottle, and all bottles were placed in the 35° C incubator. At hourly intervals thereafter, complete and deficient media were removed from replicate monolayers. The monolayers were washed twice with EBSS, a procedure later shown by titrations of the second wash fluids to have been adequate for removal of virus that had not become adsorbed to cells at the time of washing. GM was placed on the cells after the wash. The microscopic plaques which formed on the monolayers as a result of viral cytopathogenic effect during incubation at 37° C for three additional days were enumerated. Averages of the numbers of plaques which appeared on replicate monolayers are recorded in Table 4 and Figure 9.

That each plaque appearing within three days after inoculation represents one infectious particle present in the inoculum has been demonstrated by Farnham (1958), who found a linear dose-response relationship and a good agreement between observed plaque counts and the theoretical Poisson distribution expected of independently distributed particles. In the present experiment, once the experimental

TABLE 4

ADSORPTION OF HERPES SIMPLEX VIRUS TO HELA CELLS
IN PRESENCE OF COMPLETE AND DEFICIENT MEDIA

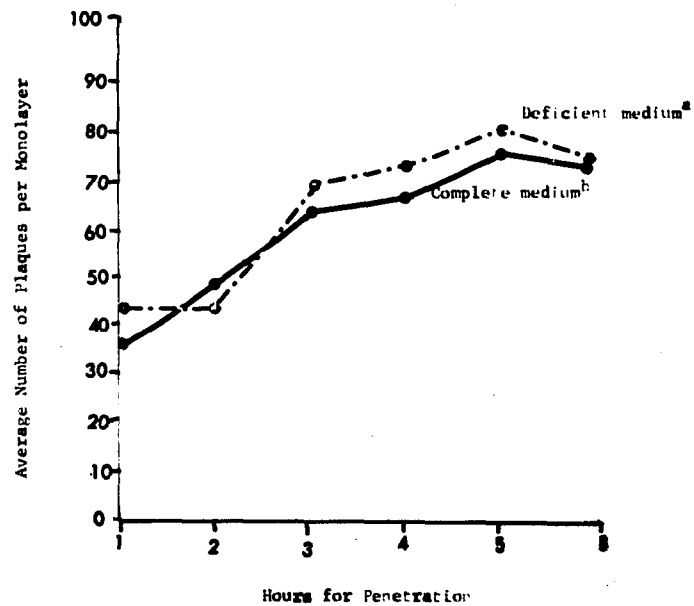
Hours for Adsorption	Number ^a of Plaques on Monolayers	
	Complete medium ^b	Deficient medium ^c
1	35	42
2	47	43
3	62	68
4	65	72
5	76	80
6	70	74

^a Number; average of plaque counts on monolayers three days following adsorption.

^b Complete medium; Eagle's basal medium containing five percent calf serum.

^c Deficient medium; Eagle's basal medium containing five percent calf serum, deficient in glucose and glutamine.

FIGURE 9
ADSORPTION OF VIRUS ON HELA CELLS
IN PRESENCE OF COMPLETE AND DEFICIENT MEDIA



- a. Eagle's basal medium less glucose and glutamine.
b. Eagle's basal medium.

media were replaced with GM there was no significant difference between the number of plaques in the monolayers supplied with deficient medium during the adsorption period and the number of plaques in monolayers furnished with complete medium during that time. Therefore, lack of viral synthesis in the deficient medium was not due to failure of adsorption of virus to cells. With this system, adsorption was maximal five hours after addition of virus, and 100 percent of the inoculated infectious units were not adsorbed.

The deficient medium did not inactivate the virus. Had it done so, fewer plaques would have developed on monolayers exposed to deficient medium during adsorption than on those monolayers that were supplied with complete medium during the adsorption period.

Another possibility which might account for lack of viral synthesis by cells in the deficient medium is that virus fails to penetrate cells in the presence of such medium. "Penetrated" virus has been defined as virus that is insensitive to inactivation by immune serum (Farnham and Newton, 1959). In order to investigate this possibility, the experiment which compared adsorption of virus in the two media was repeated with two modifications. The first change

was the decrease of inoculum from 200 to 100 TCID₅₀ of virus. This facilitated counting the plaques that developed on the monolayers. The second modification was inclusion of pooled human serum at a final concentration of ten percent in the GM added to the monolayers after the adsorption periods. As described above, this serum pool contained a high concentration of specific neutralizing antibody against herpes simplex virus. Inclusion of the serum in the GM should neutralize virus that has adsorbed to the cells before the monolayers are washed, but has not penetrated by the time GM is added. Intracellular virus is not affected by immune serum (Stoker, 1959).

Results of this experiment are depicted in Table 5 and Figure 10. These data indicate clearly that no real difference exists between the rate of cellular penetration by virus in complete medium and the rate in the deficient medium. The rate of penetration may be compared with the rate of adsorption that was found in the previous experiment. Although conclusions derived from such a comparison may be of limited value because of unknown variables between the two experiments, the data suggest that most adsorbed virus also penetrates. Under these experimental conditions, penetration seems to be slower initially than

TABLE 5

PENETRATION OF HERPES SIMPLEX VIRUS INTO HELA CELLS
IN PRESENCE OF COMPLETE AND DEFICIENT MEDIA

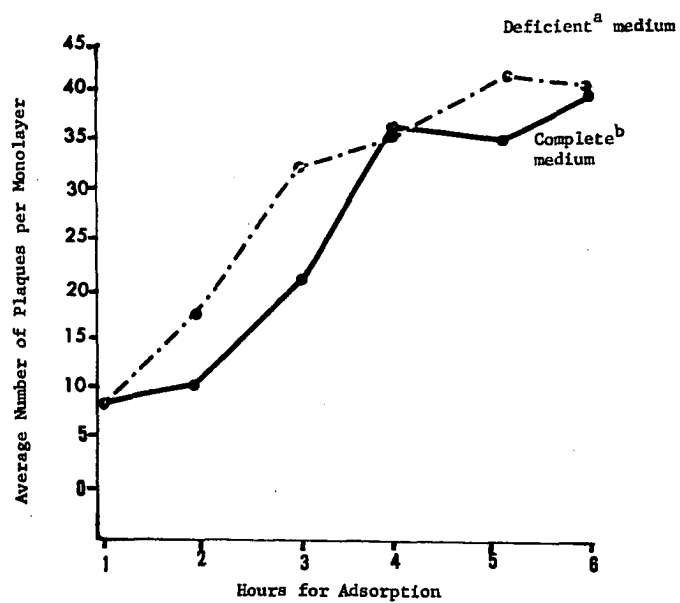
Hours for Penetration	Average Number ^a of Plaques on Monolayer	
	Complete medium ^b	Deficient medium ^c
1	8	7
2	10	17
3	22	32
4	36	35
5	35	40
6	37	38

^a Number; average of plaques counts on monolayers three days following exposure to virus.

^b Complete medium; Eagle's basal medium containing five percent calf serum.

^c Deficient medium; Eagle's basal medium containing five percent calf serum, deficient in glucose and glutamine.

FIGURE 10
PENETRATION OF HERPES SIMPLEX VIRUS INTO HELA
CELLS IN PRESENCE OF COMPLETE AND DEFICIENT MEDIA



a: Eagle's basal medium less glucose and glutamine.
b: Eagle's basal medium.

adsorption. However, within the limits of sensitivity of the test system, penetration is complete by the fourth hour.

Yet another explanation for the lack of detectable virus in glucose- and glutamine-deficient medium might be that virus, although synthesized by cells in such medium, is not released from the cells. In order to test this possibility experimentally, monolayers of HeLa cells were grown in stationary tubes with GM, washed twice in EBSS, and divided into two groups. One group received EBM containing five percent calf serum, and the other tubes were supplied with the same medium except for omission of glucose and glutamine. In each instance, after six hours the experimental medium was removed, and the tube received 4000 TCID₅₀ of virus suspended in EBSS. Following an adsorption period of one hour at 35° C, all monolayers were washed twice in EBSS, and the particular experimental medium that had been removed from each tube was placed again on that monolayer. The use of "conditioned" media avoided the addition to the cells of a fresh supply of glucose and glutamine which might be contained in the serum of unused medium.

Media were removed from groups of tubes at determined intervals for titration. The cells were washed twice with

EBSS. Subsequent test of the second wash detected no virus, which indicated that the extracellular infectious particles had been removed. After being washed, the cells were frozen quickly in an alcohol-dry ice bath and were then thawed rapidly by immersion in a 37° C water bath. After three cycles of freezing and thawing, only cellular debris was visible upon microscopic examination. This treatment did not inactivate virus significantly. A sample of viral suspension which was added to an uninfected monolayer and immediately frozen and thawed three times possessed as much infectivity as did an identical sample which was added to cells that had been disrupted previously by this treatment. Since both of these control samples possessed as much infectivity as did an identical sample that was not exposed to cellular debris, such debris apparently did not inactivate infectivity. The cellular debris of each experimental tube was suspended in GM and titrated for viral content. Results of the titrations of both extracellular and intracellular virus are shown in Table 6 and Figure 11. Each recorded titer represents an average from two tubes.

It may be noted from these data that a deficient medium did not seriously impede release of virus from the cell. At no time after 16 hours post-infection did the

TABLE 6

COMPARISON OF INTRACELLULAR AND EXTRACELLULAR VIRUS
PRODUCED BY CELLS IN COMPLETE AND DEFICIENT MEDIA

Hours Post- infection	Complete Medium ^a Amount ^c of Virus per Tube		Deficient Medium ^b Amount of Virus per Tube	
	Extra- cellular	Intra- cellular	Extra- cellular	Intra- cellular
8	0	0	0	0
16	1	0.5	0	0
24	2.5	1	0	0.5
40	2.5	1	0.5	0.5
48	2	2	1	0.5
56	2	2	1	0.5
64	2.5	1	1	0

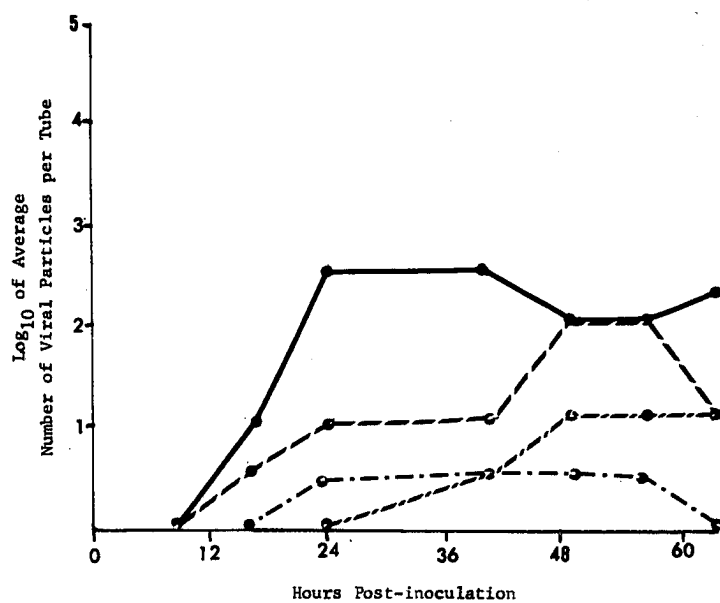
^a Complete medium; Eagle's basal medium containing five percent calf serum.

^b Deficient medium; Eagle's basal medium containing five percent calf serum, lacking glucose and glutamine.

^c Amount; $\log_{10}$ of average number of infectious particles per tube.

FIGURE 11

COMPARISON OF INTRACELLULAR AND EXTRACELLULAR
VIRUS PRODUCED BY CELLS IN COMPLETE^a AND DEFICIENT^b MEDIA



a: Eagle's basal medium.

b: Eagle's basal medium, less glucose and glutamine.

—: Extracellular virus in complete medium.

- - - : Extracellular virus in deficient medium.

- . - . : Intracellular virus in complete medium.

. . . . : Intracellular virus in deficient medium.

amount of intracellular virus in the deficient medium exceed the intracellular or extracellular amount in complete medium.

Virus apparently is released into both media soon after maturation, since infectious particles did not accumulate markedly intracellularly. Virus was detected first both extracellularly and intracellularly 16 hours after infection in the cultures with complete medium. Obviously, infectious virus had matured intracellularly and had been released in these cultures during the time between the taking of the eight and 16 hours samples. Formation of the first infectious particles by cells in deficient medium was detected eight hours later than by cells in complete medium, and virus liberated by the former cells only after an additional eight hours. Finally, from the data it is evident that much less virus is synthesized by cells supplied with deficient medium than by cells in complete medium.

Conceivably, the viral titration method employed may have detected more readily infectious particles that had been produced by cells in complete medium than those produced by cells in deficient medium. Such biased sensitivity of the method would have resulted in an apparent difference between the two media in amounts of virus synthesized although

no real difference existed. An experiment was designed to test this possibility. Tubed monolayers of HeLa cells were propagated in GM and divided into two groups. After two washes in glucose-free EBSS half the monolayers received complete EBM containing five percent calf serum, while the other monolayers were covered with this medium minus glucose and glutamine. In each case, the medium was removed after six hours and 4000 TCID₅₀ of herpes simplex virus per monolayer were allowed to adsorb onto cells. Next, the monolayers were washed twice for removal of unadsorbed virus. No virus was detected in the second washes. The original experimental medium was replaced on the original monolayers from which they had been removed. Samples of medium were taken at determined intervals and titrated for viral concentration. Parallel titrations were carried out by use of HeLa cells nourished with GM, HeLa cells covered with lactalbumin hydrolysate medium, and by inoculation of the chorioallantoic membranes of developing chick embryos. Results are presented in Table 7.

Results indicate that less virus is produced by cells in deficient medium than in complete medium, regardless of the method used for detection of the virus. The most sensitive titration system was that composed of HeLa

TABLE 7

PARALLEL TITRATIONS OF SAMPLES FOR VIRUS PRODUCTION BY CELLS IN
COMPLETE^a AND DEFICIENT^b MEDIA

Hour Post- infection	Average Viral Concentration per ml of Sample from each Medium					
	Titration with HeLa cells				Titration with chick embryo	
	In Eagle's basal medium		In lactalbumin medium			
	Sample complete medium	Sample deficient medium	Sample complete medium	Sample deficient medium	Sample complete medium	Sample deficient medium
8	0	0	0	0	0	0
16	10 ²	0	0	0	10 ^{2.1}	0
24	10 ⁵	0	10 ³	0	10 ^{3.2}	0
40	10 ⁵	10 ¹	10 ³	10 ¹	10 ^{3.9}	10 ^{0.3}
48	10 ⁴	10 ²	10 ⁴	10 ²	10 ^{4.1}	10 ^{0.9}
56	10 ⁴	10 ²	10 ⁴	10 ²	not done	not done
64	10 ⁵	10 ²	10 ⁴	10 ²	10 ^{4.3}	10 ^{0.6}

^a Eagle's basal medium.

^b Eagle's basal medium less glucose and glutamine.

cells nourished by GM. The chick embryo titration method was intermediate in sensitivity, while the HeLa cell-lactalbumin hydrolysate medium system indicated that slightly less virus was present in the samples than did the other systems. As in earlier experiments, the period after adsorption before discernable virus was released by cells in complete medium was about 16 hours. Again, virus was not detected in deficient medium as soon as in the complete medium.

To this point the investigation satisfactorily established the fact that herpes simplex virus is produced only to a slight extent by cells in glucose- and glutamine-free Eagle's basal medium. Likewise, experiments proved that the nutritional deficiency resulted in incompetency for intracellular synthesis of virus, rather than in failure of viral adsorption, penetration or release.

Glucose and glutamine were examined separately for their effects on viral proliferation. Tubes of monolayers which had been propagated in GM were divided into six groups. The cells were washed twice with EBSS without glucose and the monolayers of each group were covered by one of the following solutions: (1) complete EBM, (2) EBM without glucose, (3) EBM without glutamine, (4) EBM without either

glucose or glutamine, (5) EBSS with the usual 0.1 percent glucose and to which two mM glutamine had been added and (6) EBSS without glucose. Each of the media in which EBM was a constituent contained five percent calf serum.

Following a 12 hour incubation period for depletion of endogenous reserves of the nutrients under investigation, the test solutions were removed. Two thousand TCID₅₀ of virus were then allowed to adsorb to the cells in each tube. Control monolayers in each group were uninoculated. Following the adsorption period, each monolayer was washed twice. Except that they were devoid of serum, the wash solutions were of the same composition as those with which the washed monolayers had been supplied during the 12 hour depletion period. In this manner, unadsorbed virus was removed without addition to the monolayers of additional amounts of the nutrients under test. No virus was demonstrated in the second wash fluids. Next, the original solution which had been removed from each tube and saved was placed again in that tube. The results of this experiment are shown in Table 8.

As in previous experiments, virus was not detected in complete EBM until 16 hours after viral adsorption. Extracellular virus was demonstrated for the first time also at 16 hours from cells in EBM deficient only in glutamine.

TABLE 8

EFFECT OF GLUCOSE AND GLUTAMINE ON SURVIVAL OF HELA CELLS AND VIRAL SYNTHESIS

Medium		Hours at which Samples were taken after Viral Adsorption										
		0	8	16	24	32	40	48	56	64	72	80
EBM ^a Complete	Log ₁₀ virus per ml	0	0	1	2	2	3	3	3	4	4	3
	Control cells ^b	100 ^c	100	100	100	100	100	100	100	100	100	100
EBM, no Glucose or Glutamine	Log ₁₀ virus per ml	0	0	0	0	0	0	0	0	0	0	0
	Control cells	100	100	100	100	100	100	100	25	3	0	0

^a Eagle's basal medium.

^b Cells of uninfected monolayers maintained on same test media as infected cells.

^c Percent of cells of uninfected monolayers which appeared viable.

TABLE 8--Continued

Medium		Hours at which Samples were taken after Viral Adsorption										
		0	8	16	24	32	40	48	56	64	72	80
EBM ^a , no Glutamine	<u>Log₁₀ virus per ml</u>	0	0	1	1.5	2	3	3.5	3	2.5	2.5	1
	Control cells ^b	100 ^c	100	100	100	100	100	100	75	70	70	50
EBM, no Glucose	<u>Log₁₀ virus per ml</u>	0	0	0	0	0	0	0	0	0.5	1	2
	Control cells	100	100	100	100	100	100	100	75	10	10	10

^a Eagle's basal medium.

^b Cells of uninfected monolayers maintained on same test media as infected cells.

^c Percent of cells of uninfected monolayers which appeared virable.

TABLE 8--Continued

Medium		Hours at which Samples were taken after Viral Adsorption										
		0	8	16	24	32	40	48	56	64	72	80
EBSS ^a with Glutamine	Log ₁₀ virus per ml	0	0	0	0	0	0	0	0	0	0	0
	Control cells ^b	100 ^c	100	100	100	100	50	0	5	0	0	0.5
EBSS, no Glucose	Log ₁₀ virus per ml	0	0	0	0	0	0	0	0	0	0	0
	Control cells	100	100	50	0	0	0	0	0	0	0	0

^a Earle's balanced salt solution.

^b Cells of uninfected monolayers maintained on same test media as infected cells.

^c Percent of cells of uninfected monolayers which appeared viable.

Viral production was approximately equal in the presence of these two media until 64 hours post-adsorption, when the samples of glutamine deficient medium contained less virus. At this time, as judged by light microscopy, about 25 percent of the cells in the uninoculated monolayers maintained in the latter medium appeared to be dead. Monolayers with complete medium remained healthy throughout the experimental period.

The appearance of uninfected monolayers in EBM without glucose paralleled that of uninfected monolayers without glutamine until 64 hours post-adsorption. At this time, the former monolayers showed a decrease of about 90 percent in cell population, whereas about 70 percent of control cells in the glutamine deficient medium were still normal. Nevertheless, in the medium lacking only glucose virus was detected in the 64, 72, and 80 hours samples. The small amount of virus which was produced in this medium appeared after most of the cells appeared to be dead.

In agreement with earlier experiments, virus was not produced by cells in medium deficient in both glucose and glutamine. The first decrease in cell number of uninoculated control monolayers in this medium was observed 56 hours after viral adsorption. The initial decrease was large: around 75 percent of the cells appeared to be dead. Eight

hours later as estimated by visual examination, only three percent seemed to be alive.

It should be emphasized that the numbers of cells in uninoculated tubes appeared to be equal 48 hours after viral adsorption, regardless of whether these tubes contained complete EBM, EBM deficient both in glucose and glutamine, or EBM lacking only glucose or only glutamine. In these media the cells were indistinguishable morphologically at the 32 hours post-adsorption observation. By this time substantial amounts of virus had been synthesized by infected cells supplied with glucose containing media, whether or not the media were deficient in glutamine. In contrast, viral replication was absent during this period in glucose deficient media regardless of the glutamine content.

EBSS supported no viral multiplication even when both glutamine and glucose were present. All cells of uninfected control monolayers covered with EBSS lacking glucose and glutamine were rounded when the zero hour sample was taken. At this time all monolayers had been covered with the experimental solutions for 12 hours. The uninfected monolayers supplied with EBSS containing two mM glutamine in addition to the usual 0.1 percent glucose first appeared to be rounded at the time of the 32 hour sampling.

By 40 hours 50 percent of the control cells seemed to be dead, and eight hours later all appeared to be non-viable. A few cells still survived in the control tubes examined at the fifty-sixth hour and the eightieth hour sampling. Cells in control monolayers were indistinguishable morphologically 24 hours after viral adsorption whether supplied with complete EBM, with EBM lacking glutamine or with EBSS plus glutamine. Nevertheless, by this time virus had been produced by infected cells maintained in the first two solutions but not by those in the last solution.

The experiment illustrated that herpes simplex virus is not produced by HeLa cells which are supplied with EBM containing five percent calf serum and deficient in glucose. It was shown further that glutamine need not be added to this medium in order for virus to be synthesized. The third principal observation was that EBSS will not support viral replication in the cells even when glutamine is present. Finally, differences in amount of viral replication by cells maintained in the various media cannot be explained solely by different capacities of the media to preserve general physiological competence of the cells, if this competence be judged by cellular morphology and survival time.

It seemed important to determine whether the five percent calf serum employed in EBM contained sufficient glutamine to support normal metabolism of HeLa cells. Accordingly, the cells from one monolayer were distributed in equal numbers into three bottles. GM was added to two of these bottles, while the third received complete EBM containing five percent serum but no glutamine. Upon incubation at 37° C for six days, the cells with complete medium multiplied to produce monolayers. Only a few of the cells supplied with the deficient medium attached to the glass. These cells were extremely fusiform. Although they survived more than 220 hours, they failed to multiply.

The possibility existed that results of previous experiments had been influenced by some unknown systematic error in compounding the experimental media. Therefore, viral production in Eagle's basal medium as made in this laboratory was compared with its production in commercial EBM. A second purpose of the experiment was to determine the effect on viral synthesis of substitution of purines for glutamine in EBM. Since glutamine contributes to biosynthesis of adenine and guanine, demonstration that these purines fail to stimulate viral replication in the absence of glutamine would be evidence that the amino acid was

nutritionally non-essential for viral elaboration by the cells.

After propagation of HeLa cells in bottles until monolayers were formed, the monolayers were washed twice in EBSS. Replicate monolayers were depleted of endogenous reserves of the nutrients under test by incubation in the deficient experimental media for five hours before viral adsorption. Following this, 6.4×10^6 TCID₅₀ of virus were allowed to adsorb to each monolayer which contained from six to eight million cells. The monolayers were washed twice with EBSS, and the experimental media were added. The second wash fluids were negative upon test for virus. Replicate monolayers received complete EBM that had been obtained commercially, complete EBM made in this laboratory, commercial EBM lacking glutamine, and EBM made in this laboratory which was deficient in glutamine. Other replicate monolayers were supplied with commercial EBM lacking glutamine and supplemented with either 0.8 mg percent adenine or with 0.8 mg adenine plus approximately 0.4 mg percent guanine. All media contained five percent calf serum. Results of viral titration of samples removed at determined intervals are recorded in Table 9.

TABLE 9

EFFECT OF PURINES UPON VIRAL PRODUCTION BY HELA CELLS IN EAGLE'S BASAL
MEDIUM WITHOUT GLUTAMINE

Medium	Viral Concentration per ml of Sample at Hours Indicated after Adsorption									88
	24	32	40	48	56	64	72	80	84	
EBM ^a , complete	10 ¹	10 ¹	10 ²	10 ³	10 ⁴	10 ³	10 ^{2.5}	10 ²	10 ¹	
EBM, commercial complete	0	0	10 ¹	10 ¹	10 ²	10 ^{2.5}	10 ²	10 ¹	10 ^{0.5}	
EBM, deficient in glutamine	10 ¹	10 ²	10 ³	10 ⁴	10 ⁵	10 ⁴	10 ³	10 ²	10 ²	

EBM, commercial, deficient in glut- amine	10^1	10^1	$10^{1.5}$	10^2	10^3	$10^{3.5}$	10^3	10^3	10^3
EBM, commercial, deficient in glut- amine, with 0.8 mg % adenine	10^1	$10^{1.5}$	$10^{2.5}$	10^3	10^3	$10^{4.5}$	10^5	10^4	$10^{4.5}$
EBM, commercial, deficient in glut- amine, with 0.8 mg % adenine and approximately 0.4 mg % guanine	10^1	10^1	$10^{2.5}$	10^3	10^3	10^4	10^4	10^3	10^3

^a EBM represents Eagle's basal medium containing five percent calf serum. Unless indicated as commercial, the medium was made in this laboratory.

As in earlier experiments, virus was produced in medium that lacked only glutamine. Almost all samples from medium deficient in glutamine contained at least one log more virus than corresponding samples from complete medium. The second notable observation was that both complete and deficient media that were made in this laboratory supported viral replication somewhat better than did the corresponding commercial media. Finally, inclusion of adenine appeared to have a stimulatory effect on viral synthesis. The effect was not accentuated by presence of guanine in addition to adenine.

Two observations from this experiment seemed to be worthy of further exploration: the apparent stimulatory effect on viral production of adenine in glutamine deficient EBM, and the inhibition of viral synthesis by glutamine in EBM containing five percent calf serum. In addition to re-examination of these points, the next experiment was designed to test the effect on viral synthesis of inclusion in EBM of glycine, serine, alanine, proline, aspartic acid, and glutamic acid. These amino acids are derived partly from glutamine by HeLa cells supplied with complete EBM (Levinton et al., 1957), and they are not components of the standard medium. It appeared to be of interest to determine

whether HeLa cells synthesized more virus when relieved of the necessity of producing these metabolically essential, nutritionally non-essential amino acids.

Accordingly, an experiment was performed which was, except for one major and three minor differences, identical in design with that of the preceding study. One minor difference was that in the later experiment the cells were maintained in test media for six hours, rather than five, before adsorption. The second slight deviation from the preceding experiment was that 5×10^6 TCID₅₀ of virus, instead of 6.4×10^6 , were used as inoculum. The last small change was in the time intervals at which samples were taken. These changes in procedure were introduced for convenience in the mechanics of carrying out the experiment. The major difference between this and the preceding experiment was in the types of media used. In the present experiment, replicate monolayers received either (1) complete EBM, (2) EBM complete except for glutamine, (3) EBM with 0.8 mg percent adenine, (4) EBM with 0.8 mg percent adenine but without glutamine, (5) EBM with 0.2 mM each of glycine, serine, alanine, proline, and aspartic acid, but minus glutamine, (6) EBM with 0.2 mM of these five amino acids in addition to 0.8 mg percent adenine, but without glutamine, and (7) EBM

with the same concentration of these five amino acids as before and also 0.2 mM glutamic acid, but minus glutamine. All experimental media contained five percent calf serum. The extent of viral multiplication in cells supported by each medium is recorded in Table 10 and in Figure 12. Since no virus was found in the EBSS that had been used to wash the monolayers for the second time after viral adsorption, the infectious particles found in the samples were considered to be newly formed virus and not residual inoculum. Aliquots for cell counts were taken at the same time that the final samples for determination of viral production in infected monolayers were taken. As may be seen in Table 10, the results of the counts suggest that cells were adequately maintained for the duration of the experiment by each medium employed. Also, uninfected control monolayers supplied with each medium were examined microscopically each time a sample was removed from the infected cultures. Morphologically, the appearance of all control monolayers was identical.

The data show that cells covered with complete EBM produced practically no virus, and none was detected in EBM to which adenine had been added. Consequently, results with the latter medium are not shown in Figure 12. All media from which glutamine had been omitted not only supported cells

TABLE 10

EFFECT OF NUTRITIONALLY NON-ESSENTIAL AMINO ACIDS AND PURINES ON PRODUCTION OF
VIRUS AND SURVIVAL OF HELA CELLS

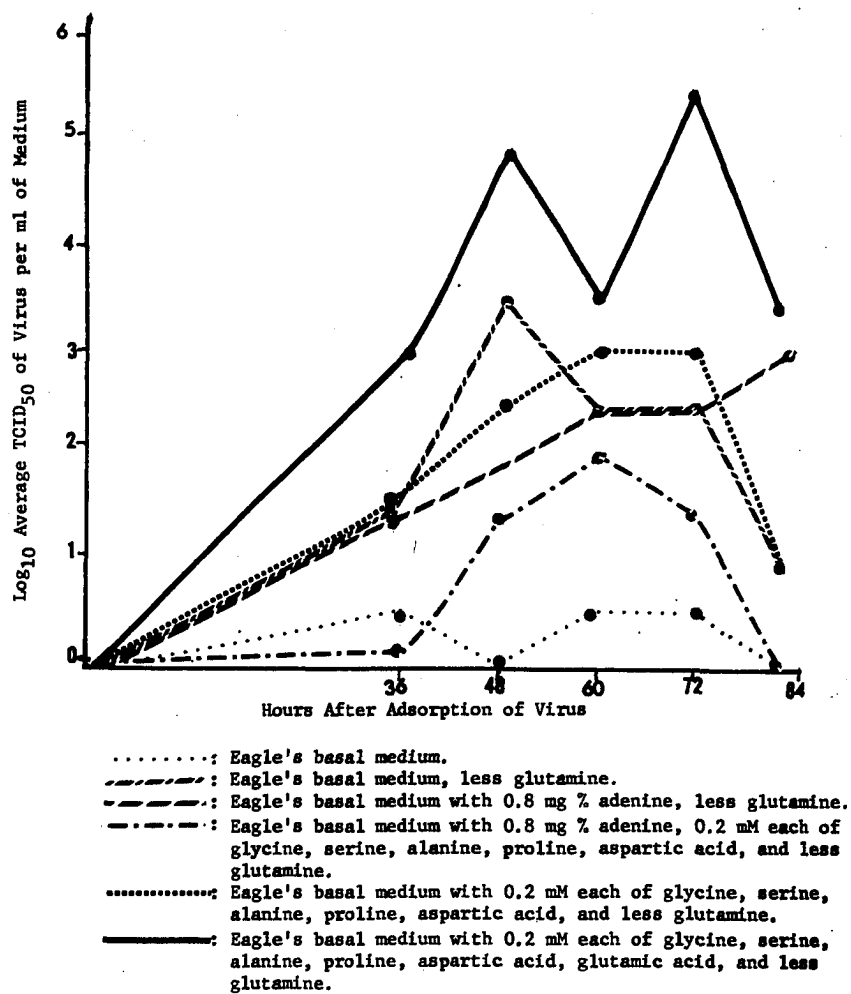
Modification of Eagle's Basal Medium		Hours at which Samples were taken after Adsorption				
		36	48	60	72	84
None	<u>Log₁₀ virus per ml</u>	0.5	0	0.5	0.5	0
	Control cells ^a	Normal ^b	Normal	Normal	Normal	4.7x10 ⁶ ^c
Glutamine omitted	<u>Log₁₀ virus per ml</u>	1.5	3.5	2.5	2.5	1
	Control cells	Normal	Normal	Normal	Normal	3.9x10 ⁶
Glutamine omitted, 0.8 mgm % adenine added	<u>Log₁₀ virus per ml</u>	1.5	2	2.5	2.5	3
	Control cells	Normal	Normal	Normal	Normal	5.5x10 ⁶
Glutamine omitted, 5 additional amino acids ^d added	<u>Log₁₀ virus per ml</u>	1.5	2.5	3	3	1
	Control cells	Normal	Normal	Normal	Normal	7.1x10 ⁶

0.8 mg % adenine added	<u>Log₁₀ virus per ml</u>	0	0	0	0	0
	Control cells	Normal	Normal	Normal	Normal	8.5x10 ⁶
Glutamine omitted, 0.8 mg % adenine and 5 additional amino acids ^d added	<u>Log₁₀ virus per ml</u>	0	1.5	2	1.5	0
	Control cells	Normal	Normal	Normal	Normal	Not Done
Glutamine omitted, 6 additional amino acids ^e added	<u>Log₁₀ virus per ml</u>	3	5	3.5	5.5	3.5
	Control cells	Normal	Normal	Normal	Normal	3.6x10 ⁶

- ^a Cells of uninfected monolayers maintained on same test media as infected cells.
- ^b Appearance of uninoculated monolayers maintained on the various media.
- ^c Cell counts made at time of last sample.
- ^d Glycine, serine, alanine, proline, aspartic acid, all at 0.2 mM concentration.
- ^e The five amino acids listed above plus glutamic acid, all at 0.2 mM concentration.

FIGURE 12

EFFECT OF NUTRITIONALLY NON-ESSENTIAL
AMINO ACIDS AND PURINES ON PRODUCTION
OF HERPES SIMPLEX VIRUS BY HELA CELLS



capable of synthesizing virus, but they allowed much more virus to be produced than did the complete medium. This characteristic of glutamine-free EBM was consistent regardless of whether adenine or the amino acids glycine, serine, alanine, proline and aspartic acid were present. In fact, cells furnished glutamine deficient EBM that contained both the purine and these five amino acids were unable to elaborate as much virus as were cells furnished with any of the other glutamine deficient media. The highest titer of virus was formed by cells nourished by EBM which lacked glutamine but to which glutamic acid had been added.

The experiment demonstrated that inclusion in glutamine deficient EBM of nutritionally non-essential purine and amino acids which are derived partly from glutamine by HeLa cells does not stimulate viral synthesis by these cells. The experiment also brought into sharp focus two intriguing questions: (1) what concentration of glutamine must be present in EBM containing five percent calf serum for inhibition of herpes simplex virus synthesis to occur, and (2) is the stimulation of viral elaboration by the inclusion of glutamic acid in glutamine-free EBM a reproducible phenomenon?

In order to answer these questions, an experiment which had the same basic design as that of the previous two

was carried out. The bottled monolayers were exposed to the test media for 7.5 hours before exposure to virus, and ten infectious particles per cell were used for adsorption. All samples of EBSS with which the monolayers were washed the second time after adsorption contained no detectable virus. The experimental media employed on the replicate monolayers were composed of Eagle's basal medium to which had been added five percent calf serum and the various concentrations of glutamine or glutamic acid indicated in Table 11 and in Figure 13.

The most striking result of the experiment is the clear confirmation of the results of previous experiments which indicated that production of virus is depressed in the presence of medium containing both five percent calf serum and glutamine. Large amounts of virus was synthesized when either 0.5 mM or no glutamine had been added to EBM containing calf serum. When one, two, four, or eight mM of glutamine were added to the serum containing medium, almost no virus was elaborated. Since no virus was synthesized by cells nourished with complete medium that contained four and eight mM of glutamine, the results with these media do not appear in the figure. Glutamic acid at 0.2 or five mM concentration substituted for glutamine only to a limited extent

TABLE 11

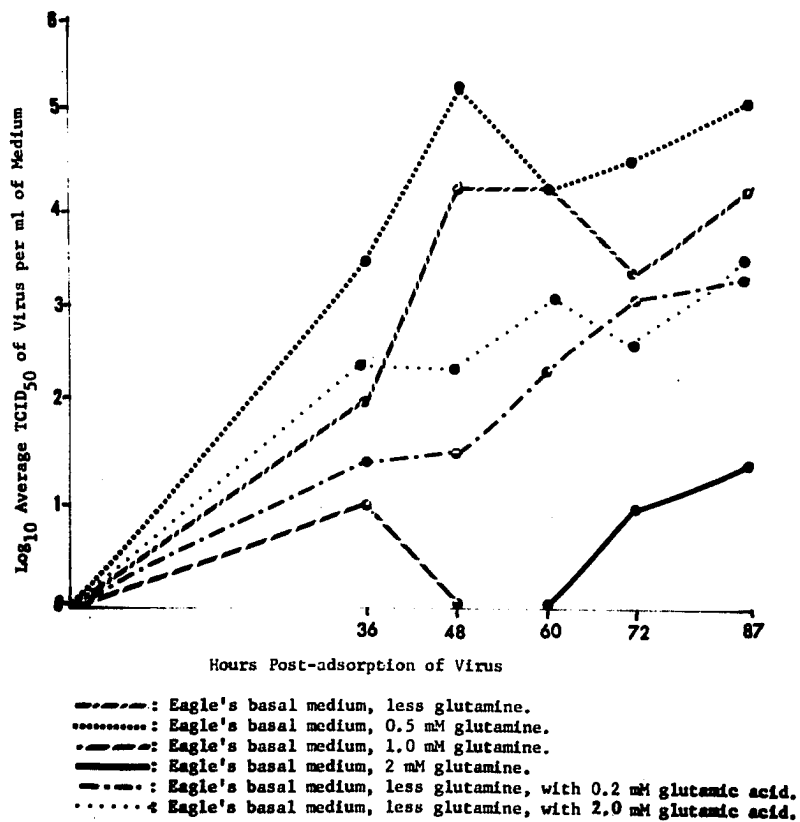
EFFECT OF GLUTAMINE AND GLUTAMIC ACID ON VIRAL PRODUCTION
BY HELA CELLS IN EAGLE'S BASAL MEDIUM

Medium	TCID ₅₀ of Virus per ml Medium at Hour Indicated ^a				
	36	48	60	72	87
EBM ^b , no glutamine	10 ²	10 ^{4.3}	10 ^{4.3}	10 ^{3.6}	10 ^{4.3}
EBM, 0.5 mM glutamine	10 ^{3.5}	10 ^{5.3}	10 ^{4.3}	10 ^{4.6}	10 ^{5.3}
EBM, 1 mM glutamine	10 ¹	0	0	0	0
EBM, 2 mM glutamine	0	0	0	10 ¹	10 ^{1.5}
EBM, 4 mM glutamine	0	0	0	0	0
EBM, 8 mM glutamine	0	0	0	0	0
EBM, no glut- amine, 0.2 mM glutamic acid	10 ^{1.4}	10 ^{1.5}	10 ^{2.3}	10 ³	10 ^{3.3}
EBM, no glut- amine, 2 mM glutamic acid	10 ^{2.3}	10 ^{2.3}	10 ³	10 ^{2.5}	10 ^{3.5}

^a Time in hours after viral adsorption when sample was taken.

^b Eagle's basal medium.

FIGURE 13
EFFECT OF GLUTAMINE AND GLUTAMIC ACID
ON VIRAL PRODUCTION BY HELA CELLS IN EAGLE'S BASAL MEDIUM



in suppression of viral multiplication when the bovine serum was present also.

The results of this experiment were compatible with the hypothesis that viral replication is suppressed when glutamine is present above a certain concentration. This critical concentration may be exceeded by the glutamine content of the five percent calf serum added to medium which already contains 0.5 mM or more of glutamine. The next experiment was performed to investigate this possibility.

Various concentrations of serum were employed. Two modifications of the preceding experiment were made: the monolayers were washed three times in EBSS rather than twice to free them of traces of growth medium before the test media were added and, for adsorption, the viral inoculum for each bottle was suspended in four ml instead of six ml. By use of the smaller volume for adsorption, it was hoped that the number of cells initially infected would be increased. Unfortunately, areas of the monolayers became somewhat dried during the period of adsorption, despite periodic rocking of the bottles for distribution of the fluid over the cells. As a result, microscopic observation of the monolayers immediately following addition of the experimental media revealed that all of them contained areas

composed of rounded, granular cells. Table 12 and Figure 14 depict composition of the test media employed in this experiment together with the extent of viral synthesis by cells maintained in these media.

As may be seen from the data, the medium containing ten percent serum was found to yield slightly more virus than did that containing five percent serum. An additional observation was that cells nourished by EBM deficient in glutamine and containing ten percent serum synthesized virus to high titer, as did complete EBM without serum. In both cases, the amounts of virus produced were greater than in cells maintained in EBM containing both serum and glutamine.

The preceding experiment demonstrated that virus was produced in high titer by monolayers in the absence of serum. This observation permitted a more direct approach to clarification of the interrelations between serum and glutamine in viral synthesis. In the next experiment two modifications were introduced: an increase to six ml of the volume of viral suspension employed for adsorption, and a change in the times at which samples were taken. Viral production by cells that were supplied with EBM without serum, EBM without glutamine, EBM without both serum and glutamine and complete EBM were

TABLE 12

EFFECT OF VARIOUS CONCENTRATIONS OF CALF SERUM ON PRODUCTION
OF HERPES SIMPLEX VIRUS BY HELA CELLS

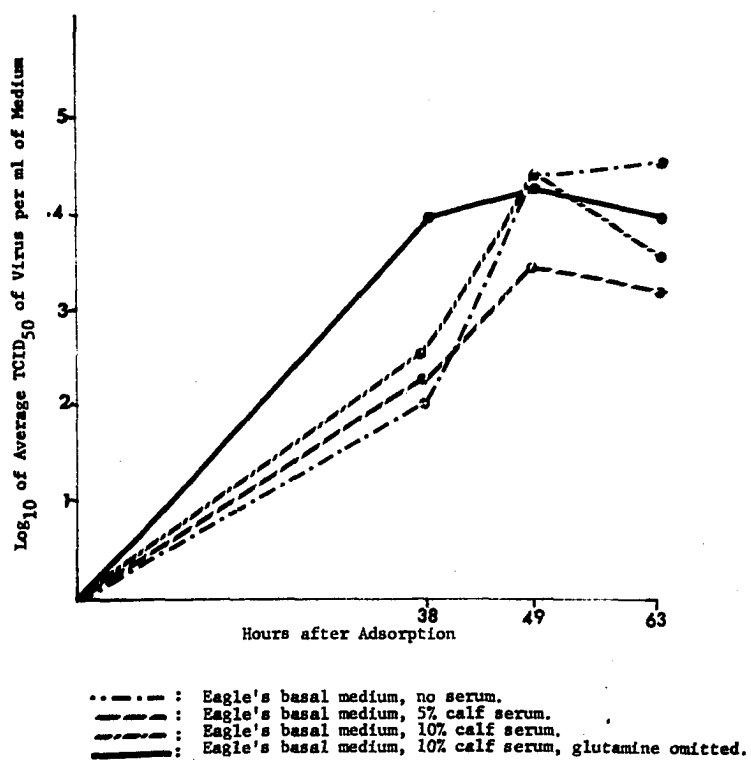
Medium	TCID ₅₀ of Virus per ml Medium at Hour Indicated ^a		
	38	49	63
EBM ^b , with serum omitted	10 ²	10 ^{4.4}	10 ^{4.5}
EBM, with 5% calf serum	10 ^{2.3}	10 ^{3.5}	10 ^{3.2}
EBM, with 10% calf serum	10 ^{2.5}	10 ^{3.5}	10 ^{3.6}
EBM, with 10% calf serum, glutamine omitted	10 ⁴	10 ^{4.3}	10 ⁴

^a Time in hours after viral adsorption when sample was taken .

^b Eagle's basal medium.

FIGURE 14

EFFECT OF VARIOUS CONCENTRATIONS OF CALF SERUM
ON PRODUCTION OF VIRUS BY HELA CELLS



compared. In addition, determinations were made of the amount of virus elaborated by cells nourished either with complete EBM or with EBM without glutamine when the serum added to the medium had been inactivated at 56° C for 30 minutes. When serum was included in the medium, it was at five percent concentration. The results of this experiment are shown in Table 13 and Figure 15.

As in earlier experiments, much more virus was detected in media that were deficient either in glutamine or in serum than in medium containing the two mM glutamine of complete EBM in addition to five percent calf serum. Thus, complete EBM supported replication of approximately a hundred times less of virus than did EBM without glutamine when the serum of the two media had not been inactivated. Approximately the same ratio occurred with comparable media which contained inactivated serum.

Substantial amounts of virus were synthesized by cells in medium devoid of both serum and glutamine. This viral production occurred despite inability of the medium to support adequately the over-all metabolic competence of the cells as evaluated by microscopic examination. For example, at the time the 60 hour post-adsorption sample was taken approximately 70 percent of the cells appeared to be dead.

TABLE 13

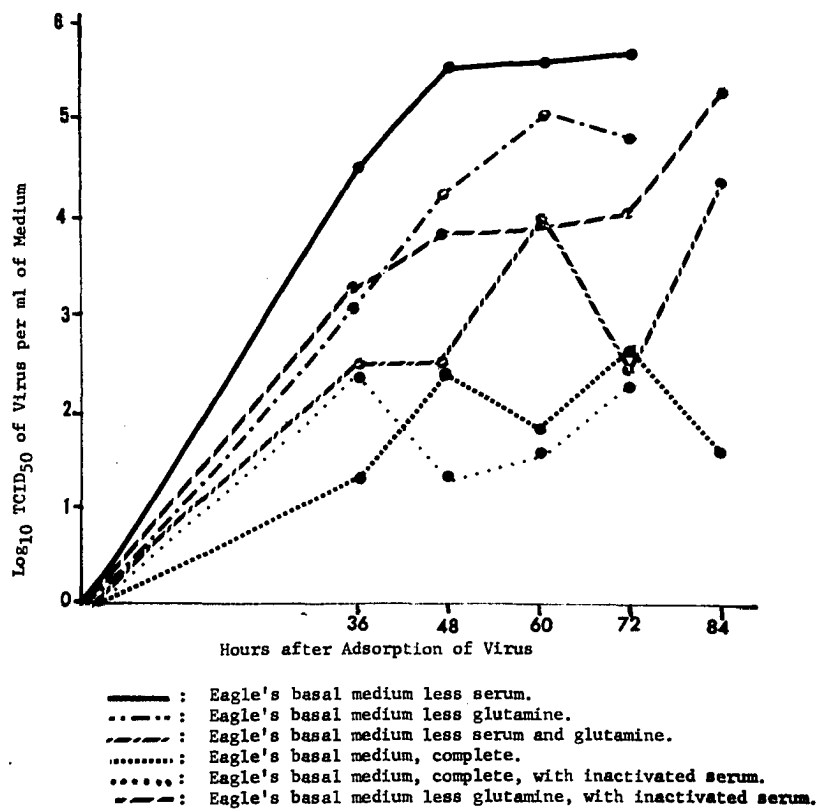
EFFECT OF GLUTAMINE AND INACTIVATED SERUM ON VIRAL
SYNTHESIS BY HELA CELLS

Medium	TCID ₅₀ of Virus per ml Medium at Hour Indicated ^a				
	36	48	60	72	84
EBM ^b , no serum	10 ^{4.5}	10 ^{5.5}	10 ^{5.5}	10 ^{5.6}	not done
EBM, no glutamine	10 ³	10 ^{4.2}	10 ⁵	10 ^{4.8}	not done
EBM, no serum or glutamine	10 ^{2.4}	10 ^{2.4}	10 ⁴	10 ^{2.4}	10 ^{4.3}
EBM, complete	10 ^{1.3}	10 ^{2.3}	10 ^{1.8}	10 ^{2.5}	10 ^{1.6}
EBM, complete, inactivated serum	10 ^{2.4}	10 ^{1.3}	10 ^{1.5}	10 ^{2.2}	not done
EBM, inactivated serum & no glutamine	10 ^{3.3}	10 ^{3.8}	10 ^{3.5}	10 ⁴	10 ^{5.25}

^a Time in hours after viral adsorption when sample was taken.

^b Eagle's basal medium.

FIGURE 15
EFFECT OF GLUTAMINE AND INACTIVATED
SERUM ON VIRAL SYNTHESIS BY HELA CELLS



Surviving cells, when free of viral cytopathogenic effect, were fusiform. Nevertheless, the viral concentration of this sample was 10^4 per ml. Twenty-four hours later the titer of virus had increased even further.

Inactivation of the serum contained in the media had no apparent effect on the production of virus. As much virus was synthesized in EBM containing uninactivated serum as in that with inactivated serum. Also, EBM lacking glutamine and containing uninactivated serum supported viral production that was at least equal in amount to that of cells with the same medium in which the serum had been inactivated.

The previous experiment had indicated that virus was produced by cells furnished with medium which lacked both glutamine and bovine serum. It seemed desirable to determine the reproducibility of this observation. The design of the following experiment was exactly as in the preceding test. Media employed and results obtained are shown in Table 14 and in Figure 16.

Again, virus was produced in the absence of both glutamine and serum. In the preceding experiment, the 36 hour sample of medium with neither glutamine nor serum contained $10^{2.4}$ TCID₅₀ of virus per ml; the 36 hour sample

TABLE 14

VIRAL PRODUCTION IN EAGLE'S BASAL MEDIUM CONTAINING NO
SERUM AND VARIOUS CONCENTRATIONS OF GLUTAMINE

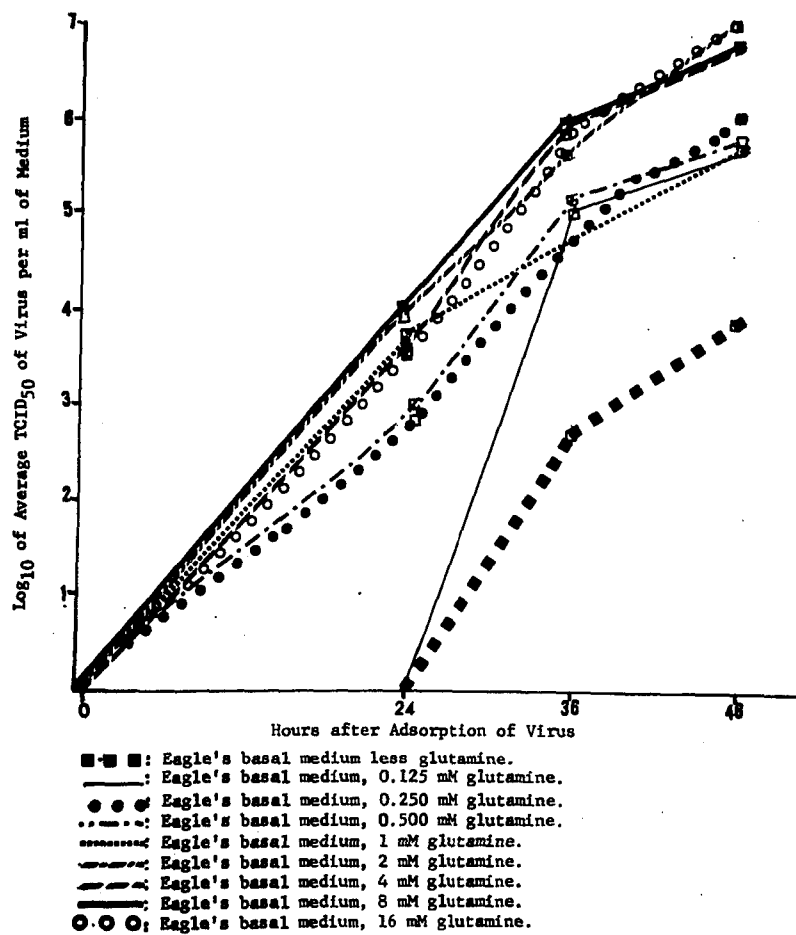
Medium	TCID ₅₀ of Virus per ml Medium at Hour Indicated ^a		
	24	36	48
EBM ^b , no glutamine	0	10 ^{2.7}	10 ^{3.5}
EBM, 0.125 mM glutamine	0	10 ⁵	10 ^{5.7}
EBM, 0.250 mM glutamine	10 ^{2.7}	10 ^{4.8}	10 ⁶
EBM, 0.5 mM glutamine	10 ^{2.7}	10 ⁵	10 ^{5.7}
EBM, 1 mM glutamine	10 ^{3.7}	10 ^{4.7}	10 ^{5.7}
EBM, 2 mM glutamine	10 ⁴	10 ^{5.7}	10 ⁷
EBM, 4 mM glutamine	10 ^{3.7}	10 ⁶	10 ^{6.7}
EBM, 8 mM glutamine	10 ⁴	10 ⁶	10 ^{6.7}
EBM, 16 mM glutamine	10 ^{3.7}	10 ⁶	10 ⁷

^a Time in hours after viral adsorption when sample was taken.

^b Eagle's basal medium, containing no serum.

FIGURE 16

VIRAL PRODUCTION IN EAGLE'S MEDIUM
CONTAINING NO SERUM AND VARIOUS CONCENTRATIONS OF GLUTAMINE



in the present test had $10^{2.7}$. Presence of glutamine in the medium increased viral production approximately a hundred-fold. In the 48 hour sample the medium with 0.125 mM of glutamine supported viral replication to a final concentration that was approximately equal to the concentrations found at that time in media containing as much as 1 mM glutamine. However, the synthesis of virus within the first 24 hours after infection was at least ten times less in cells nourished with media containing as much as 0.5 mM glutamine than in cells with media containing 1 mM or more. At 36 hours, no great difference was apparent in viral production by cells with media to which glutamine had been added in any concentration. Forty-eight hours after viral adsorption, samples of media from such monolayers revealed no marked difference in titer of virus. Sixteen mM glutamine, eight times the ordinary concentration in EBM, did not suppress viral multiplication.

Uninoculated monolayers were maintained in all media during the experimental period. As with control cells in previous experiments, these monolayers were treated by washing, inoculation, removal of samples and in all other respects exactly as their infected counterparts. The only abnormal appearance seen microscopically in control cells

during the experiment was a slight rounding and granularity of cells in media containing no glutamine at time of removal of 24, 36, and 48 hour samples, and a similar appearance of cells with 0.125 mM glutamine at 36 and 48 hours.

The differences between viral production by cells with complete EBM and with glutamine-free EBM were analyzed statistically. Titers of 72 hour samples were used because such samples were taken in more of the experiments which compared viral synthesis in these two media than were other samples. In order that the samples be comparable, only data from experiments in which the cells had been covered with test media from five to $7\frac{1}{2}$ hours before viral inoculation were included. These data, shown in Table 15, have appeared in the descriptions of the experiments in which they were determined (Tables 9, 10, 11, and 13).

A Student's t-test was applied (Snedecor, 1957). Under the null hypothesis, a t-value (degrees of freedom = 5) was calculated to be 4.1, and such a value has a chance probability of less than .01 associated with it. This probability level indicates that the null hypothesis may be rejected and that HeLa cells probably produce more virus in the absence of glutamine than in the presence of it under the experimental conditions which were used.

TABLE 15

STUDENT'S t-DISTRIBUTION^a ANALYSIS OF VIRAL PRODUCTION BY HELA CELLS
IN EAGLE'S BASAL MEDIUM WITH AND WITHOUT GLUTAMINE

Table from which Data were Taken	Log ₁₀ TCID ₅₀ of Virus per ml of Sample ^b		Difference D=X ₁ -X ₂	Deviation d=D- $\bar{d}$	Squared Deviation d ²
	Deficient medium X ₁	Complete medium X ₂			
9	3.0	2.5	0.5	-1.2	1.44
9	3.0	2.0	1.0	-0.7	0.49
10	2.5	0.5	2.0	0.3	0.09
11	3.6	1.0	2.6	0.9	0.81
13	4.8	2.5	2.3	0.6	0.36
13	4.0	2.2	1.8	0.1	0.01
Totals			10.2	0	3.20
Mean			$\frac{10.2}{6} = 1.7 = \bar{d}$		$\frac{3.20}{5} = 0.64 = sD^2$

$$s\bar{d}^2 = \frac{0.64}{6} = 0.17; \quad s\bar{d} = 0.41; \quad t_{(5)} = \frac{1.7-0}{0.41} = 4.1, \text{ P less than } 0.01$$

^a Computations patterned after and symbols taken from Snedecor (1957).

^b 72 hour samples.

CHAPTER IV

DISCUSSION

Effect of Glucose on Proliferation of Herpes Simplex Virus by HeLa Cells

The results of this investigation show that HeLa cells fail to synthesize herpes simplex virus in the absence of an oxidizable carbohydrate. Of 128 separate cellular monolayers which were deprived of glucose and then infected, only 18 synthesized detectable virus. Of the latter, four had not been maintained in glucose deficient medium before infection, and eight had been in such medium for only six hours. Although the time required for complete depletion of endogenous carbohydrate reserves by HeLa cells in medium lacking such compounds has not been determined experimentally, it is possible that the time may exceed six hours. The remaining six of the 18 monolayers produced virus even though inoculation had occurred after 12 hour starvation in glucose deficient medium. However, the small amount of virus synthesized by these cells first appeared 64 hours

after inoculation. At this time, 90 percent of the cells in the monolayers appeared to be no longer living. Autolysis of dead cells could have released enough carbohydrate for support of the limited viral production that occurred.

It is possible that glucose serves only to maintain the integrity of the host cell so that viral synthesis may occur, without playing a direct role in that synthesis. Many cells died in glucose deficient medium at a time when those in complete medium were healthy. Nevertheless, virus was produced by cells with complete medium and not by those with deficient medium during the time that the numbers of cells in the two media were entirely comparable. During this period, cells in the glucose deficient medium were indistinguishable morphologically from those of the complete medium. The use of vital stains for detection of subtle differences between the two populations was not attempted because the value of vital staining methods is limited in cell culture (Paul, 1959). Manometric techniques were not utilized because cellular concentrations greater than those found in monolayers must be obtained for recognition of respiratory changes which are so subtle as to produce no morphological effect. The mechanics of obtaining such concentrations alter cellular metabolism drastically, and small

initial differences between populations are sure to be masked. Although use of isotopically labeled compounds could have revealed subtle effects of glucose-deficient medium on cellular metabolism, whether such effects were pertinent to the suppression of viral proliferation would remain unanswered. For the above reasons, conditions of the populations were judged by direct microscopic observation.

There is further indication that glucose was not involved simply in allowing cells to survive until they produced virus. In other experiments described, cells in medium which lacked both serum and glutamine synthesized appreciable amounts of virus at a time when they were in what appeared to be an advanced state of morphological degeneration. Conversely, cells in glucose deficient medium were unable to produce infectious particles at a time when they appeared to be normal.

It was established experimentally that cellular adsorption and penetration of virus occurred as readily in medium without glucose as in complete medium. Since there was no intracellular accumulation of infectious particles in the presence of deficient medium, the lack of viral production by the cells was not attributable to inhibition of viral release. Conceivably, viral formation by cells in deficient medium could have been unrecognized because the particular

titration system employed for detection of virus was for some reason more sensitive to infectious particles which had been formed by cells in complete medium. The use of several titration systems in parallel indicated that this was not the case. The sum of evidence, therefore, suggests that glucose enters directly into the intracellular synthesis of herpes simplex virus by HeLa cells.

These cells are considered to be facultatively anaerobic (Gifford and Syverton, 1957). Whether the energy for synthesis of herpes simplex virus is derived by anaerobic or by aerobic oxidation is not known with certainty. Barron (1932) presented evidence which suggested the tricarboxylic acid cycle was not important in synthesis. He noted a marked inhibition of the activity of succinodehydrogenase in the brains of rabbits which were infected in vivo with herpes simplex virus. Similar information is meager in the case of other viruses. Moulder et al. concluded in 1953 that the energy for multiplication of feline pneumonitis virus in cultures of chick embryo yolk sac was generated by aerobic oxidation. The same source of energy is believed to support replication of influenza virus in cultures of chick embryo tissue (Magill and Francis, 1936; Eaton and Perry, 1953; Ackermann, 1951a; Ackermann and Johnson, 1953).

Conversely, the energy for formation of poliovirus is thought to be derived from glycolysis in HeLa cells (Gifford and Syverton, 1957) as well as in cultures of human amnion and of monkey kidney cells (Becher et al., 1958). The energy for production of Newcastle disease virus by MCN cells seems to come from glycolysis (Green et al., 1958).

In addition to serving as energy source, intermediary metabolism of glucose may supply specific compounds which are required directly or indirectly for viral synthesis. Eagle and Habel suggested in 1956 that glucose carbon might contribute to the nucleic acids of poliovirus produced by HeLa cells in Eagle's basal medium. This suggestion was prompted by the observation that approximately a hundred times less virus was formed when glucose was omitted from the medium, and that the effect of glucose was not on fixation of virus to the cell. Darnell and Eagle (1958) reported that fructose at higher concentration than glucose could substitute for glucose in elaboration of poliovirus. Galactose, ribose, pyruvate, ribose-5-phosphate, and combination of ribose and pyruvate caused only a minor increase in viral production even at concentrations in excess of those which permitted growth of HeLa cells in glucose-free medium.

While fixation of herpes simplex virus in the present investigation was shown to be unaffected by absence of glucose, even less of this virus than of poliovirus is formed when the sugar is omitted from the cellular medium. The biochemical reactions leading to synthesis of virus are without doubt endergonic. Study of production of herpes simplex virus by HeLa cells in the presence of various enzyme inhibitors and intermediary compounds of the glycolytic and tricarboxylic acid cycles and of the pentose phosphate pathway is feasible. Such study may elucidate the reactions that supply the necessary energy and may detect shifts produced by viral infection in utilization of the customary cellular metabolic pathways. The possibility of developing effective chemotherapy from application of such information is apparent. In addition, isotopically labeled carbohydrates may be employed to find what contribution is made by carbon of such compounds to viral protein and nucleic acid.

Early in the investigation, attempts were made to prepare a relatively nutrient-free viral pool for use in later nutritional experiments. Reason for the failure of those attempts is understood more fully in light of the vital role demonstrated later for glucose in viral replication.

In order to make such a pool, a viral inoculum of suitable potency must be employed, and the optimal time intervals after infection must be determined both for replacement of GM with salt solution containing no glucose and for harvest of virus. However, it was decided that this amount of preliminary experimentation was unjustified by the slight benefit such a pool might contribute to the present study. In the experiments that had been anticipated for this study, as little as 0.1 ml of a highly infectious viral pool would be sufficient to obtain the desired high virus to cell ratio for the bottle monolayers. The negligible quantity of nutrients which might be contributed by this volume would be diminished even further by thorough washing of the monolayers after the viral adsorption period. Furthermore, differences that might be noted in production of virus by monolayers which were nourished with various media could hardly result from a viral inoculum uniform for all of the monolayers. These predictions appeared to be fulfilled by results of later experiments.

An uncloned strain of HeLa cells was used throughout the present investigation. It was assumed that employment of a strain which had been derived from a single cell would offer no advantage over use of an uncloned strain, since

HeLa cells, in common with all other forms of life, undergo continuous mutation. The extent of genotypic heterogenicity found within a cloned strain after several generations should be similar to that of uncloned strains because the same pressures of population dynamics and selection may be reflected by both.

Effect of Glutamine on Proliferation of
Herpes Simplex Virus by HeLa Cells

The results obtained by this investigation leave no doubt that depletion of the free glutamine in HeLa cells does not inhibit synthesis of herpes simplex virus by the cells. Substantial quantities of virus were produced in every instance in which the 2 mM glutamine ordinarily present in EBM were omitted. This was observed with 22 separate tube monolayers and 32 distinct bottle monolayers. Statistical analysis indicated that the increase in viral titer of 72 hour samples which was observed upon omission of glutamine from EBM could be expected to occur by chance alone less than one in a hundred times.

The possibility existed that enough glutamine to support viral elaboration was contributed by the five percent calf serum. The fallacy of this assumption was shown by

significant viral synthesis on the part of six monolayers in EBM lacking both serum and glutamine. In experiments which tested the role of glutamine in viral elaboration, cells were maintained for at least six hours upon glutamine deficient medium before exposure to virus. This period is sufficient to eliminate intracellular reserves of the amino acid (Darnell and Eagle, 1958).

While the above proof is sufficient demonstration that glutamine is not nutritionally necessary for elaboration of herpes simplex virus by HeLa cells, additional, indirect evidence on this point was obtained. Glutamine carbon and nitrogen enter into purine synthesis (Salzman et al. 1958). Also, glutamine carbon is found in the six amino acids which are nutritionally non-essential for HeLa cells (Levinton et al. 1957). Therefore, if the presence of glutamine contributed to viral multiplication, the contribution could perhaps depend upon the conversion of the glutamine to these end products, which might be the immediate precursors of viral protein and nucleic acids. Were this true inclusion of these amino acids and purines in EBM from which glutamine had been omitted might be expected to stimulate cellular viral production. However, this possibility was not confirmed experimentally. No increase in viral multiplication was observed upon

substitution for glutamine of these substances in various combinations. Lack of permeability of HeLa cells was not a factor since these cells are permeable to both adenine (Salzman and Sebring, 1959) and the amino acids (Levinton et al., 1957). The observation that the presence of purines in the medium had no noticeable stimulatory effect on viral multiplication is of some interest, since nucleic acids are such prominent constituents of viruses. Results of the substitution of glutamic acid for glutamine are of particular interest. It is known that HeLa cells contain the enzyme, glutamine synthetase, which converts glutamic acid to glutamine (Eagle et al., 1956). This reaction does not occur rapidly enough for support of cellular multiplication in the absence of glutamine unless very high concentrations of glutamic acid are present. Also, it does not occur to an extent that affects viral production since presence of glutamic acid did not prevent the enhanced viral synthesis obtained with glutamine deficient cells, a phenomenon to be discussed below.

The demonstration that glutamine is not required for synthesis of herpes simplex virus by HeLa cells gains additional interest when a report of Eagle and Habel (1956) is recalled. They observed that synthesis of poliovirus by HeLa

cells was decreased approximately 200 fold in EBM when glutamine was omitted. They speculated that glutamine may furnish nitrogen for synthesis of viral nucleic acid, and believed that the amino acid participates directly in viral replication. In addition, Eagle and Habel found that virus was produced almost as well by EBSS to which 2 mM glutamine had been added as by complete EBM. It was found in the present investigation that no herpes simplex virus is formed under such conditions. Definition of the precise role played by this compound in multiplication of HeLa cells and of polio-virus in these cells that is not required for synthesis of herpes simplex virus would be most instructive concerning the biochemistry of viral infection.

Eagle and co-workers (1959) demonstrated that omission of a single nutritionally essential amino acid from the basal medium of Eagle resulted in cessation of net protein synthesis by normal HeLa cells. Nevertheless, herpes simplex virus was shown in the present study to be formed by these cells in the absence of nutritionally essential glutamine. Therefore, a supply of glutamine must have been available to the cells which was adequate to support formation of viral protein, yet insufficient for cells to multiply. Perhaps this glutamine arises from glutamic acid. The glutamic acid may be derived

by transamination of ketoglutarate from the tricarboxylic acid cycle or from metabolism of the histidine that is included in EBM (White et al., 1959). Formation of some glutamine from the glutamic acid could be accomplished by the small concentrations of glutamine synthetase present in the cells. Alternatively, the glutamine for viral synthesis may come from catabolism of host cell protein. Some protein of the cell that is to produce virus may be degenerated as in normal metabolism, or the source of this protein may be other cells which die in the monolayer. The protein need not be degraded completely to the amino acid level; Eagle (1955b) showed that the amino acids necessary for survival and growth of HeLa cells could be replaced by dipeptides containing these amino acids. Employment of radioactive isotopes might allow evaluation of the relative importance of various sources of the amino acid. At any rate, once small amounts of glutamine are available to the infected cell it is likely that the reactions leading to viral synthesis have priority over the normal demands of the cell for the amino acid.

Comparisons of viral replication by cells which were maintained in commercial media with that in cells supplied with corresponding media made in this laboratory proved the

latter to be satisfactory. In fact the locally prepared media supported synthesis of virus somewhat better than did the corresponding commercial media.

Effect of Sub-optimal Conditions for Cellular Metabolism
on Proliferation of Herpes Simplex Virus

Study of data obtained from this investigation leads to the unexpected conclusion that more herpes simplex virus is produced by cells maintained under certain sub-optimal conditions than by cells that are entirely healthy. In 36 of 38 monolayers maintained on glutamine deficient EBM, the viral production was approximately one hundred times greater than in monolayers with the complete medium. Production was as great in the complete medium in only two instances. Omission of serum from medium which covered 18 monolayers stimulated viral synthesis to approximately the same extent as did omission of glutamine. In the experiments in which purines as well as nutritionally non-essential amino acids were included in the glutamine-free EBM, the medium supported less viral synthesis than did glutamine deficient media not containing these compounds. That the former medium may be considered to be more nearly nutritionally complete than any of the other glutamine deficient media tested is perhaps more than coincidence.

Stimulation of viral replication that resulted from use of incomplete EBM was not as marked in one series of experiments as in others. This was attributable perhaps to the fact that in the former series of experiments, all monolayers had inadvertently undergone exposure to unfavorable environmental conditions during the period of viral adsorption. This fact was shown by the appearance of the cells.

More virus was elaborated by cells in the presence of EBM that lacked calf serum than by cells in the presence of the complete medium. This is not explainable by the presence of inhibitory heat labile substances that may be contributed by the serum to the medium. Inactivation of the serum did not affect the results. This information is pertinent in view of the demonstration by Finkelstein and co-workers (1958) that herpes simplex virus is sensitive to the properdin system.

The idea was considered that perhaps the suppression of viral production by the complete medium reflected presence of inhibitory concentrations of glutamine. Eaton and associates demonstrated in 1951 that synthesis of myxoviruses in chick embryo tissue culture was inhibited by basic amino acids. Glutamine contained in the calf serum added to EBM in most of the present experiments would supplement the two mM of glutamine already present in the medium. That this was

not the explanation in these experiments was shown by two approaches. According to the first, if the glutamine of the serum were inhibitory, an increase in serum concentration of the medium might enhance the suppression of viral elaboration. However, when ten percent serum was used in medium containing two mM glutamine viral production did not differ markedly from that found in the presence of five percent serum only. Also, use of ten percent serum in medium from which glutamine had been omitted did not change the amount of virus produced from that formed by cells with such medium that had only five percent serum.

The second approach was more decisive. A concentration of glutamine as high as 16 mM was shown to exert no inhibition on viral multiplication in EBM lacking serum. For the five percent serum in the medium to have raised the final glutamine concentration as much as 14 mM would have required the serum to have contained 280 mM. This is scarcely in the realm of possibility since human serum usually contains less than 0.6 mM of the amino acid (White et al., 1959).

Glutamine and serum protein both are essential for normal propagation of HeLa cells (Eagle, 1955a). Presumably, they are necessary for optimal cellular metabolism. That healthy cells are more susceptible to viral infection than

are poorly nourished ones is a widely held opinion. It is reasoned that since the virus depends for replication on the metabolic machinery of the host cell, an active cell is capable of producing more virus than is one with sub-optimal metabolism. This idea may not be completely correct since the present investigation has shown that viral synthesis is enhanced by omission from the medium of nutrients essential for continued multiplication of the cells. While a degree of cellular metabolism is certainly essential for viral proliferation, the defense potential of a wholly normal cell may be higher than that of one maintained under certain sub-optimal conditions. For example, nutritional and other requirements for synthesis by cells of interferon have yet to be elucidated fully.

Observations of Kovacs (1956a, 1956c) indicate that enzymes of monkey kidney cell cultures appear in the medium when the latter is nutritionally deficient. The more deficient the medium, the more rapid is the loss of a variety of intracellular enzymes. Kovacs showed that cell disintegration which occurred in the deficient medium did not account for all of the enzyme increase in the fluid phase. He suggested that depletion of enzymes may render the cells more susceptible to viral infection and capable of viral production,

since under such conditions it may be easier to direct the cells into the required specific synthetic pathways.

Andrewes and Carmichael observed as early as 1930 that recurrent herpes only occurred in persons whose serum contained neutralizing antibodies specific for the virus. These observations and the fact that herpes did not seem to spread from person to person but seemed to be provoked by non-specific stimuli such as fever, menstruation, and emotional up-set, led Doerr (1938) even to suggest "that herpes is not an infectious agent which is maintained by a chain of infection but that it is endogenously generated in the human organism." However, Dodd et al. (1938) found that isolation of the virus could be accomplished routinely from the mouths of infants suffering from primary stomatitis. The difficulties were resolved completely by the demonstration by Burnet and Williams (1939) that such infants also developed neutralizing antibodies during convalescence from the stomatitis. It is recognized at present that the virus appears to become latent in the cells of the host (Scott, 1956). Despite the presence of antibodies in the circulation, it causes local damage under circumstances altering the physiology of the host. Nevertheless, the immediate cause of activation

remains to be elucidated. Results of the present investigation suggest that the answer may lie at least partly in alteration of the metabolism at the cellular level. Further experimental exploration of this interesting possibility by employment of both cell culture and in vivo techniques appears to be in order.

In preliminary experiments for determination of the minimal amount of calf serum which must be present in EBM for support of normal cellular metabolism the incidental observation was made that a few cells survived longer than did the majority in populations nourished by media containing sub-optimal amounts of serum. Apparently, certain members of the cellular populations were better suited for survival under such conditions. This survival advantage may be genotypic or it may depend on the phase of growth in which a particular cell exists at the time the shortage of serum occurs. A few cells, therefore, live longer under the specific adverse circumstance than do the majority. Perhaps the survival period is prolonged even longer by utilizable metabolites made available upon autolysis of the dead cells in the monolayers.

Growth Characteristics of Herpes Simplex

Virus in HeLa Cells

Of considerable interest is the information obtained during the present investigation concerning the growth characteristics of herpes simplex virus in HeLa cells. The growth of this virus in the chorioallantois of the developing chick embryo has been studied by several workers (Schaffer and Enders, 1939; Scott et al., 1953; Wildy, 1954; Modi and Tobin, 1954; Yoshino and Taniguchi, 1957). The advantages of relatively homogeneous monolayers or suspensions of cells in culture have attracted several workers to these systems for determination of growth characteristics of the virus. Gostling and Bedson (1956) employed trypsinized suspensions of chick embryo cells, while Kaplan (1957) utilized rabbit kidney cell cultures. Perhaps the most exhaustive study of the growth characteristics of herpes simplex virus in cell culture has been by the English group, (Stoker, 1959; Stoker and Ross, 1958; Farnham, 1958). Information obtained by these workers may be compared with results of the present study since both utilized HeLa cells and the HF strain of virus.

Adsorption. Stoker and Ross (1958) reported that when HeLa cells were exposed to herpes simplex virus suspended in fluid one mm deep, 50 percent of the virus was adsorbed by the end of the first hour. Thereafter, the rate became slower and approached the rate of thermal inactivation. Farnham (1958) found a similar rate of adsorption. The present investigation indicated that 70 percent of the added infectious particles became attached by the end of the first hour and that at least 20 percent remained unadsorbed even after six hours at 35° C when the experiment was terminated. Thus, there is close agreement among the different workers in the adsorption rates, especially when such variations as volume and temperature of the suspending medium are considered.

Penetration. Data from the present investigation showed that roughly half of the virus that had adsorbed by the first hour had penetrated the cell by that time. The ratio of penetrated to adsorbed virus was the same at the end of the second hour. However, beginning at the fourth hour essentially 100 percent of adsorbed virus had penetrated. These results are in substantial agreement with those reported by Stoker (1959).

Time of Appearance of Infectious Virus. Intracellular virus was not detected eight hours after inoculation but was present at the time of the next sampling eight hours later. Stoker (1959) reported that new infectious particles were first found within HeLa cells nine hours after infection. In agreement with the work of Stoker, extracellular infectious particles were detected first in the present investigation no earlier than 16 hours after infection.

Intracellular Non-infectious Phase. In the lytic cycle of bacteriophage the occurrence of an eclipse period, during which no infectious particles are found within the infected bacterial cell, has been established beyond doubt (Doermann, 1952; Anderson and Doermann, 1952). An eclipse period has been found for nearly every virus that has been adequately tested (Prince, 1958). Stoker (1959) believes that herpes simplex virus also undergoes an eclipse phase. The existence of such a phase is consistent with results of the present investigation. Disruption of infected cells revealed no mature virus intracellularly until 16 hours after adsorption.

Release of Virus. The pattern of release of animal viruses from cells depends largely upon the particular virus involved. In the case of cells infected with poliomyelitis virus, mature infectious particles remain in the host cell

until a threshold is reached, whereupon the majority of the virus is released suddenly into the medium (Melnick et al., 1957). The suddenness of the release is reminiscent of bacterial lysis by intracellular mature phage (Ellis and Delbruck, 1939a). A second pattern of release is illustrated by chick embryo cell cultures infected with Western equine encephalomyelitis virus. Mature virus is formed and released into the medium almost as soon as it is formed (Rubin et al., 1955). The present investigation indicates that release of herpes simplex virus from HeLa cells follows the latter pattern, since there was no evidence of intracellular accumulation of infectious particles.

Stoker (1959) presents evidence that virus, in addition to infecting cells by release into the medium, may spread directly from cell to cell in a monolayer culture upon the fusion of contiguous cells. This hypothesis is strengthened by results of the present study. In experiments which examined adsorption and penetration of infectious particles, viral plaques were observed to enlarge progressively in the presence of high titer herpes simplex virus antiserum in the medium.

CHAPTER V

SUMMARY

The effect of glucose and glutamine on the production of herpes simplex virus strain HF by HeLa cells was determined by a study which employed more than 10,000 individual cellular monolayers. Omission of glucose from Eagle's basal medium resulted in marked curtailment of viral synthesis by the cells several hours prior to their morphological alteration which occurred in this deficient medium. However, viral adsorption, penetration and release were shown to occur as readily in the case of cells in the deficient medium as by cells in complete medium. Therefore, the curtailment of viral synthesis in the medium which lacks glucose appears to result from failure of intracellular synthesis of infectious particles. Nevertheless, virus was not formed by these cells when they were maintained by a simple salt solution containing glucose and glutamine in the concentrations present in Eagle's basal medium.

Inclusion of glutamine in the basal medium of Eagle was not mandatory for production of herpes simplex virus by HeLa cells. When glutamine was omitted from the medium, cells produced much greater quantities of virus than when the complete medium was employed. This difference was statistically significant at the 0.01 level. The effect was not attributable to glutamine per se since high concentration of the amino acid did not depress viral synthesis in medium which contained no serum. Addition to the glutamine deficient medium of amino acids and purines which are nutritionally non-essential for growth of HeLa cells did not increase further the amount of virus produced. Omission of serum from otherwise complete medium resulted in increased viral production comparable to that obtained when glutamine alone was omitted. Although less virus was found when neither serum nor glutamine were included in the medium than when only one of these substances was omitted, this environment supported formation of more virus than did medium that was nutritionally complete.

Adsorption and penetration of herpes simplex virus were essentially complete within three hours under the experimental conditions employed. The virus entered an eclipse phase within eight hours after cellular penetration. This

eclipse phase did not persist for eight hours. Observations indicated that infectious particles are released from the cells soon after formation and do not accumulate intracellularly.

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