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REPLICATION OF HERPES SIMPLEX VIRUS IN A METABOLICALLY IMBALANCED SYSTEM

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LAWRENCE ALLEN CHITWOOD

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REPLICATION OF HERPES SIMPLEX VIRUS IN A

METABOLICALLY IMBALANCED SYSTEM

APPROVED BY:

E. C. Bracken

L. V. Scott

F. G. Felton

M. R. Shettar

H. N. Kirkman

DISSERTATION COMMITTEE

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REPLICATION OF HERPES SIMPLEX VIRUS IN A METABOLICALLY IMBALANCED SYSTEM

CHAPTER I

INTRODUCTION

Hormones are potent metabolic regulators, but the actual modes of action are virtually unknown. These regulators, in an *in vivo* situation, are in delicate balance with one another; some act synergistically and others antagonistically to control metabolism. A survey of literature reveals few papers concerned with the *in vitro* influence of hormones on virus replication. Although *in vitro* studies do not imply homologous conditions to *in vivo* studies, it was anticipated that a study concerned with the effects of hormones on tissue culture cells infected with virus might provide some insight into virus-cell interactions. Before any studies could be instigated concerning the influence of hormones on virus infected cells, it was necessary to establish effects of hormones on non-infected cells. Three hormones (insulin, growth hormone, and chorionic gonadotropin) were selected for initial screening purposes.

Early studies were designed to determine effects of concentration and time of addition of these hormones (when added singly or in combination) on cellular proliferation. Results of these investigations

suggested that effects of growth hormone or insulin warranted additional study. Since virus replication is so intimately associated with cellular metabolism, a hormone which induces greater changes in metabolism might have a greater effect on virus replication. Due to its broad spectra of activity, growth hormone, which influences carbohydrate, fat, and protein metabolism, was the only hormone chosen for detailed study. In fact, this hormone induces so many physiological effects that the term "somatotropin" has been proposed (Dorfman, 1962). Due to poor availability of human somatotropin, some initial studies utilized somatotropin of bovine origin. Although the bovine hormone is not considered to be active in primates, activity was noted in an in vitro system removed from the immunological defense mechanism, although quantitatively less than with hormone of human origin.

Results of the effects of somatotropin on tissue culture cells and tissue culture cells infected with virus suggested that somatotropin induced a metabolic imbalance in the cells. Since these studies did not provide direct evidence of the mode of action of the hormone, the effects of an antimetabolite (p-fluorophenylalanine) with a better known mechanism of action were examined in the same system. It was believed that somatotropin and the anti-metabolite might induce similar results.

The purpose of this research was to study some of the effects of somatotropin and p-fluorophenylalanine on the replication of herpes simplex virus in an in vitro system. This was approached by a number of independent but correlated methods which included biochemical, morphological and virological studies. The effects of somatotropin (and several other hormones) and p-fluorophenylalanine on the production of

herpes simplex virus were determined. Biochemical studies were directed towards determining the utilization of D glucose and the content of cellular protein under various experimental conditions. Morphological investigations included a comparison of effects by somatotropin and p-fluorophenylalanine on infected and non-infected cells.

CHAPTER II

REVIEW OF LITERATURE

Effects of Hormones on Cells and Cells Infected with Virus

Of the voluminous amount of literature available concerning effects of hormones, only those which pertain to modes of action at the cellular level and effects on replication of virus at the cellular level will be detailed in this review.

Parathyroid Hormone

This hormone, which is associated with calcium metabolism, decreases the amount of adsorption of herpes simplex virus onto HEp-2 cells if cells are pretreated with hormone 3 to 24 hours prior to infection, but has no effect when the hormone is added after infection (Roizman, 1962a).

Thyroid Hormone

Thyroid hormone has been reported to have an uncoupling effect of oxidative phosphorylation on mitochondria isolated from mammalian cells (Hoch and Lipmann, 1953). In tissue culture systems, Halevey and Avivi (1960) noted that thyroxine and triiodothyronine stimulated glucose uptake and lactic acid production. Various types of media modified

these effects. Several papers have appeared concerning the role of this hormone in cells infected with virus. Eaton et al. (1956) noted a slight stimulation of influenza PR-8 proliferation. Tissue culture cells pretreated with thyroid extract adsorb herpes simplex virus more rapidly than untreated cells, but the titer of infectious virus produced is not appreciably altered, according to Roizman (1962b). He speculated that thyroid and parathyroid hormones may influence the course of virus infections by a modification in the number of receptors available for virus attachment.

Sex Hormones

Kalter et al. (1951) reported that testosterone, a protein anabolic agent, stimulates influenza virus proliferation in vivo, whereas removal of this hormone by castration results in a decrease of virus proliferation. They suggested that virus proliferation may be related to protein metabolism of the host.

Progesterone and β-estradiol apparently exhibit a cellular specificity and concentration effects on cells grown in vitro. These hormones have little effect on the propagation of chicken embryo fibroblasts at low concentrations, but at high levels of concentration there appear to be toxic or inhibitory effects. Proliferation of HeLa cells appears to be stimulated by progesterone (Kawada, 1959). Progesterone and β-estradiol act synergistically on HeLa cell proliferation (Kawada, op. cit.), whereas testosterone and progesterone appear to act as antagonists (Someya, 1960).

Uptake of C^{14} glycine into tissue culture cells of human

endometrium is accelerated by β-estradiol. However, rate of uptake is dependent not only on presence of hormone, but is greatly influenced by age of the culture and frequency of change of media (Robertson et al., 1961).

Cortisone and Related Compounds

It has been suggested that cortisone increases gluconeogenesis in animals, resulting in a negative nitrogen balance due to enhanced transamination processes (Fisher and Fisher, 1959). In vivo studies by Szentivanyi et al. (1961) suggested that cortisone may increase transport of amino acids at the cellular level, which, due to competition for a limited supply of one or more amino acids could induce a secondary response of reduced protein synthesis at the organism level. High concentrations of hydrocortisone result in an increase of glycolysis and a decrease of respiration in HeLa cells, whereas low concentrations stimulate respiration and energy production. These data suggested to Grossfeld (1959) that hydrocortisone stimulates anaerobic metabolism. Hydrocortisone increases the size of HeLa cells, decreases cellular proliferation, and increases content of protein, RNA, and DNA per cell (Snell and Bass, 1960). Extensive vacuolization has been noted in HeLa cells when grown in the presence of this compound (Kline et al., 1957).

Studies by Wheeler et al. (1961) suggested that quantities of herpes simplex virus (HSV) produced in HeLa cells are not influenced by 1, 5, or 10 mcg of hydrocortisone per ml of media. HeLa cells infected with HSV in the presence or absence of cortisone produce the same amount of lactic acid, but cells in the presence of this hormone produce, in

addition, large amounts of acetate, pyruvate, and succinate (Fisher and Fisher, 1959). When cells are grown with or without cortisone and infected with HSV, specific activity of lactic acid from utilization of C^{14} labelled glucose is the same. Glucose labelled in the anomeric carbon gives rise to highly radioactive CO_2, whereas labelling in the number six position gives rise to only a small amount of label. Cortisone does not appreciably affect this pattern of labelling (Fisher and Fisher, 1951).

Cortisone has a definite enhancing effect on vaccinia infections in vivo (Bugbee et al., 1960). Studies by Holden and Adams (1962) indicated that hydrocortisone induces a more extensive cytopathic effect and a greater release of vaccinia in L cells than untreated cells. As suggested by investigations by Siegel and Beasley (1955), time of addition of hormone is important for effect of virus-cell interaction. When cortisone and vaccinia are introduced simultaneously into HeLa cells, a delay in cytopathic effect is noted, but addition of cortisone prior to infection induces an acceleration in cytopathic effect. Similar effects have been noted with poliovirus in the same system. Basing their observations upon amount of intracellular glucose present within hydrocortisone treated HeLa cells infected with HSV, Greenberg and Stewart (1961) have suggested that effect of hydrocortisone is one of partial return of stressed cells to a more normal physiological function.

Insulin

Studies utilizing partially purified serum have indicated that insulin stimulates the proliferation of HeLa cells (Leiberman and Owe,

1959). Insulin increases the amount of infectious poliovirus produced in a variety of tissue culture cells. The amount of increase is dependent on concentration of hormone and to a lesser extent on type of cell system employed (Likar and Wilson, 1959).

Growth Hormone (Somatotropin)

Isolation of growth hormone from bovine pituitary extracts by Li et al. (1945) furnished investigators with a preparation which, devoid of contaminating hormones, stimulated research on the biological activity of this hormone. Extensive reviews on the multiplicity of physiological actions of growth hormone have appeared (Raben, 1959, Ikkos and Luft, 1960, and Beck et al., 1960) and therefore, only the papers directly pertaining to the present studies will be reviewed.

Effect of growth hormone has been characterized by protein anabolism and increase in total body mass and skeletal growth. Moon et al. (1962) investigated effects of human growth hormone (HGH) on growth rate of human tissue culture cells by measuring protein content and nuclear multiplication as indices of growth. These studies indicated that HGH causes an increase in nuclear multiplication which is proportional to concentration and length of incubation of hormone. The hormone stimulates total protein synthesis, but not to the same extent that nuclear multiplication is stimulated, which results in a protein content per cell less than control cells. These data suggested that nuclear protein synthesis could occur at the expense of cytoplasmic protein synthesis. Morphological examination of cells in presence of hormone suggested a decrease in cytoplasmic material.

Studies on uptake of C^{14} glycine by microsomal ribonucleoprotein particles of rat liver have indicated that growth hormone stimulates incorporation of the labelled amino acid into protein (Balis et al., 1958). Prudden et al. (1956) investigated the effects of growth hormone and insulin upon synthesis of proteins in perfused livers and noted that growth hormone stimulates uptake of glycine into protein to a greater extent than growth hormone plus insulin, or insulin alone.

Effects of Protein Antimetabolites on Virus Replication

A number of studies have appeared in the literature in recent years concerning effects of protein antagonists on virus replication. Many of these investigations have been directed toward disassociating events which occur during the eclipse or latent period. An early study by Ackermann et al. (1954) indicated that the amino acid analogue, p-fluorophenylalanine (FPA), is effective in inhibiting synthesis of poliovirus in HeLa cells, but only if the compound is added during early stages of infection. Subsequently, temporal relationships of this compound have been studied in a number of other systems.

Zimmerman and Schafer (1960) studied effects of FPA added to chicken embryo cells at various times after infection with fowl plague virus. These studies disassociated S-antigen, hemagglutinin, and infective virus formation. Due to the competitive nature of FPA with phenylalanine, these studies indicated that events which are preliminary to virus proliferation, such as synthesis of virus precursors, are preserved after FPA addition. Similar investigations by Wilcox and Ginsberg (1961) with type 5 adenovirus indicated that synthesis of virus can be

inhibited during the last two to three hours of the eclipse or latent period. Parallel inhibition of soluble complement fixing antigens, toxin, and infectious virus formation suggested that these antigens represented virus precursors.

When FPA was added to adenovirus infected with tissue culture cells six to twelve hours post infection, infectious virus formation and the normally increased synthesis of DNA, RNA, and protein antigens were prevented. However, if the analogue was added 15 hours post infection all macromolecules were synthesized but infectious virus was not assembled. These findings were interpreted as suggesting that at least two phases of protein synthesis are essential during formation of infectious virus: the first, between six and twelve hours post infection, is associated with an increase in enzymes required for DNA and RNA synthesis; and the second, between 14 and 18 hours after infection, is concerned with synthesis of virus-specific antigens which are dependent upon synthesis of RNA and DNA and are probably subunits of the virus particle (Wilcox and Ginsberg, 1963, and Flanagan and Ginsberg, 1962).

Inhibitors of protein synthesis have indicated that replication of T_2 phage DNA requires a preliminary period of protein synthesis (Kornberg *et al.*, 1959). In theory, inhibition of virus replication by inhibitors of protein synthesis can be attributed to interference in production of enzymes essential for synthesis of virus macromolecules and/or synthesis of non-enzymatic proteins, i.e., structural proteins. Low concentrations of FPA inhibit maturation of western equine encephalomyelitis and poliovirus, whereas high concentrations inhibit both

maturation and infectious RNA formation (Wecker et al., 1962 and Levintow et al., 1962). The appearance of infectious RNA and virus antigen are simultaneously inhibited, which implies that virus protein itself is necessary for either formation or preservation of virus RNA as an infectious entity.

The inhibitor of protein synthesis, puromycin, like FPA, inhibits incorporation of lysine C^{14} into cellular protein. Upon removal of either inhibitor, infectious RNA accumulates at a rate which is identical to control cultures, suggesting that infectious RNA may occur without synthesis of novel enzymes. Moreover, the presence of either of these antimetabolites immediately interrupts synthesis of infectious RNA once it has been initiated, which would seem to indicate that continuing synthesis of a non-enzymatic protein is required for synthesis or preservation of infectious RNA. Wecker (1963) concluded from these studies that protein which is required for appearance of infectious RNA is not an enzyme, but is a structural protein, i.e., the virus protein. Other alternatives to their conclusion are that enzymatic proteins are produced within a very short time following removal of the inhibitor or that some proteins are formed in the presence of the antimetabolites.

Studying effects of FPA on incorporation of C^{14} leucine and p^{32} into chicken embryo cells infected with fowl plague virus, Scholtissek and Rott (1961) reported that FPA does not influence incorporation of C^{14} leucine into cellular protein or p^{32} into cellular RNA. However, they noted that the s-antigen synthesized in the presence of FPA and labelled with C^{14} leucine is not incorporated into intact virus to any appreciable degree. They concluded that the faulty s-antigen

which incorporates FPA and is serologically active remains locked in the nucleus or that it does not fit into the intact virus and is excluded from incorporation into it as a virus subunit.

By adding FPA to tubes of replicate tissue culture cells at various times after infection with vaccinia virus, Salzman et al. (1963) established that synthesis of virus protein appears to precede formation of infectious virus by no more than thirty to forty-five minutes or may possibly coincide with it. This protein seems to be the rate limiting step in formation of infectious vaccinia virus. Virus protein formed in absence of synthesis of DNA (inhibited with 5-fluorodeoxyuridine) is not available for synthesis of virus when DNA synthesis is resumed by addition of thymidine. These results would tend to show that de novo synthesis of protein is required at time of synthesis for DNA for the formation of infectious virus.

Growth and Development of Herpes Simplex Virus (HSV)

Various strains of HSV have been reported to produce a proliferative, non-proliferative, or giant cell (polykaryocyte) response in tissue culture systems (Gray et al., 1958 and Scott and McLeod, 1959), but as studies of Crandell (1959) suggested, the type of lesion produced is dependent not only on strain of virus but also the type of cell culture and size of inoculum. Infection of Maben cells (adenocarcinoma of lung) with HSV produced acid slowly without typical cytopathic effect. Cells become round and granular without formation of plaques (Coleman and Jawetz, 1961).

It would appear that the virus-cell relationship is influenced

not only by strain of virus and type of cell, but as studies of Farnham and Newton (1959) have indicated, yield of virus is very dependent on temperature and hydrogen ion concentration of suspending media. Adsorption of virus is largely independent of temperature, whereas penetration and growth decrease at temperatures less the 37° C. These studies also suggested that maximum yields of virus are obtained when pH of media is 7.0 to 7.5. Yields of virus appeared to be maximum at 32° C rather than 37° C, which was attributed to high rate of thermal decay of this virus. A number of conflicting reports have appeared in the literature in recent years concerning the rate of thermal inactivation of HSV. Although rates of inactivation appear to vary with strain of virus, type and pH of suspending media, and method of assay, in all cases it appears to follow first order reactions with a half life of approximately two to three hours at 37° C (Scott and McLeod, 1959).

Size of macroplaques of HSV in FL human amnion cells are dependent on temperature, type and concentration of serum (Hoggan et al., 1960 and Hoggan et al., 1961). Maximum plaque size is attained after four days at 37° C. Presence of 0 to 10 mM glutamine appears to have little effect on plaquing of HSV in FL cells (Hoggan et al., 1961).

Intracellular distribution of HSV in embryonic liver cells was investigated by Ackermann and Kurtz (1952). A majority (80 per cent) of virus was recovered within the cytoplasm with 16 per cent being associated with mitochondria. Results of these studies suggested that mitochondria may have some function in the development of this virus.

Numerous studies of growth of HSV have been thoroughly

reviewed by Stoker (1959) and therefore, will not be detailed in this review of literature.

Growth studies (Scott et al., 1953) associated with parallel observations on development of the intranuclear inclusion indicated that a profound disturbance occurs within the nucleus during the eclipse period. Virus replication appeared to be essentially complete prior to development of the eosinophilic Feulgen-negative inclusion. By sampling at various times after infection, Gray and Scott (1954) noted that the percentage of virus within the nucleus varies inversely with the total amount of virus and number of hours after infection; a negligible amount of virus is associated with the nucleus 12 hours post infection.

Fluorescent antibody studies of infected animal tissues and tissue culture cells (Lebrun, 1956 and Hoggan et al., 1961) have revealed that viral antigen makes its first appearance as small spherical spots in the nucleus which increase slowly in quantity and then appears within the cytoplasm in large amounts. During terminal stages of infection, at time of inclusion formation, little or no antigen could be detected within the nucleus. Cytochemical changes associated with infection of HeLa cells by HSV have been investigated by Love and Wildy (1963). These studies suggested that within one half hour after infection small ribonucleoprotein particles increase in size within the nucleoli and are subsequently extruded into the nucleoplasm. Within the nucleoplasm they have been termed B bodies. Approximately three to four hours after infection the A body (inclusion) begins to form and displaces B bodies and the nucleolus. Minute granules containing RNA, DNA, and non-histone

protein begin to appear within A bodies approximately six hours after infection.

Ross and Orlans (1958), by combining fluorescent antibody and acridine orange staining techniques, indicated that HeLa cells infected with HSV show complete breakdown of particulate nuclear DNA, which is deposited as diffuse DNA in the perinuclear region of the cell. Diffuse DNA appeared prior to antigen suggesting that accumulations of diffuse DNA may be a necessary step in synthesis of this virus.

Single step growth studies (Russell et al., 1964) with HSV have indicated that complement fixing antigens begin to increase between the first and second hours after infection and continue to increase up to nine hours post infection before remaining constant in amount. Infective virus increases exponentially between five and nine hours post-infection. Between nine and twenty-two hours post-infection, infective virus appears to remain constant.

By studying release of HSV from solitary HeLa cells, Wildy et al (1959) were able to show that the greatest release of virus occurred between 16 and 34 hours after infection. Although large inocula were used to infect cells, only 19 to 35 per cent of cells were liberating virus 28 to 35 hours post-infection. These studies also suggested that liberation of virus in one cell occurs over a period of hours with a scattering in time of release from different cells.

Metabolic Changes in Cells Induced by Infection with Herpes Simplex Virus

Biochemical studies (Green, 1962) of virus infected cells have provided substantial information concerning events which occur prior to

and during infectious virus formation. Many of these studies have been directed toward determinations of alterations in cellular metabolism, expressed as increases or decreases of cellular products, and uptake of various precursor molecules. Although it is tempting to compare data from one study to another, certain problems are encountered when such analogies are made. 1. Inasmuch as it is impossible to infect an entire population of cells at the same time, one is generally confronted with a mixed population of infected and non-infected cells. 2. Biochemical patterns of non-dividing cells in maintenance medium do not necessarily reflect those of actively growing cells in growth medium. 3. In many cases, infection of cells by virus results in accumulations of cellular debris. Therefore, net increases or decreases of metabolic products may vary when expressed in terms of a unit volume or on a per cell basis. 4. Uptake of isotopes into cells infected with virus may reflect not only alterations in rate of synthesis, but also changes in cellular permeability, turnover rates and exchange reactions.

Energy Metabolism

An in vivo study by Ackermann and Francis (1950) indicated that the activities of two oxidative enzymes, alpha-ketoglutaric acid oxidase and succinic acid oxidase, decreases in herpetic heart infections, suggesting that some aberrant change may occur in energy metabolism during infection. Fisher and Fisher (1961) investigated the utilization of glucose in HSV infected tissue culture cells. These studies indicated that infection results in increased accumulations of labelled lactic acid. Acetate and pyruvate were unlabelled or labelled

only in trace amounts. Glucose 1-C^{14} gave rise to highly labelled CO_2. However, glucose 6-C^{14} gave rise to CO_2 with low activity. These data indicated that infection results in the stimulation of the phosphogluconate oxidative pathway.

Nucleic Acid Metabolism

Newton and Stoker (1958) determined DNA content of HeLa cells at various times after exposure to a high multiplicity of herpes virus. These studies suggested that DNA content rises six to nine hours after infection, prior to onset of infectious virus formation. The DNA content, which is confined to the nucleus, doubles their normal content within 72 hours after infection. Microspectrophotometry of Feulgen stained preparations (Wildy et al., 1961) have likewise indicated a net increase of DNA per nucleus. However, Russell et al. (1964) were not able to detect any net changes in DNA during a 22 hour period with more than 50 per cent of the cells infected.

Autoradiography studies have indicated that infection of cells with HSV results in decreased rate of H^3 thymidine incorporation (Wildy et al., op. cit.). However, a report by Nii et al. (1961) showed that infection of cells with HSV results in increased uptake of H^3 thymidine.

In the same article, Wildy and his group reported that prelabelling cells with H^3 thymidine resulted in an increase in intracellular deoxyribonucleotide metabolic pools after infection, due to breakdown of host nuclei acid. If H^3 thymidine was added to cells at time of infection, label appeared distributed uniformly throughout the nucleus with only a small amount of label within the marginated

chromatin. However, in cells labelled prior to infection, label appeared mainly in the marginated chromatin (Munk and Sauer, 1964).

DNA nucleotydyl transferase (DNA polymerase EC 2.7.7.7.) and RNA nucleotydyl transferase (RNA polymerase EC 2.7.7.6) greatly increased in activity shortly after infection (Russell et al., 1964). The greatest percentage of increase in DNA nucleotydyl transferase is found in the nuclei (90 per cent) and in the mitochondria-microsomes (195 per cent) (Keir and Gold, 1963).

Prelabelling cellular DNA with P^{32} and subjecting it to density gradient centrifugation has established that a dense DNA increases in proportion to total labelled DNA from 2.5 per cent at five hours to 5 per cent at six hours and 9 per cent at seven hours. It has been estimated that the time required to duplicate a molecule of herpes virus DNA is no more than 40 minutes and to encapsulate after synthesis of DNA to be 120 to 150 minutes (Roizman et al., 1963). Chang and Liepins (1962) investigated degradation of thymidine in virus infected cells. They noted that 20 to 60 per cent of thymidine 2-C^{14} is liberated as $C^{14}O_2$ following infection with a lipovirus (including HSV) in a variety of cell lines.

Newton and Stoker (1958) were not able to demonstrate any significant changes in RNA content of infected cells.

Protein Metabolism

The studies of Russell et al. (1964) have indicated that total protein content of cells remains constant during a 22 hour period with more than 50 per cent of cells infected. Complement fixing antigens

appeared between the first and second hours after infection and continued to increase in amount to the ninth hour before coming constant.

Electron Microscopy of Herpes Simplex Virus

During recent years the electron microscope has been utilized to delineate virus particles from their intracellular environment, to determine mensural relationships of virus particles to infectivity, and to study fine structure of the virus.

Coriell et al. (1950) described the elementary body of herpes simplex derived from egg or vesicle fluid as a sphere, usually with a dense center, but in some instances the centers were absent, resulting in doughnut-shaped forms. Evidence that these bodies were actually herpes simplex virus was based upon the premise that: 1. the size was consistent with that obtained by filtration and centrifugation; 2. there was positive correlation between numbers of particles and infectious units and; 3. the morphology of particles representing different strains of virus remained the same. Morphology of HF strain (Reagan et al., 1953), propagated in mice, proved to be identical to that reported by Coriell et al. (1950).

Studies of thin-sectioned material have indicated that nuclei of infected tissue culture cells contain dense particles, 30 to 40 mu in diameter, surrounded by a single membrane, 70 to 100 mu in diameter. Most of the virus particles observed in cytoplasm are surrounded by second outer membranes, 120 to 130 mu in diameter, implying that synthesis occurs within the nucleus and maturation within the cytoplasm (Morgan et al., 1954).

Temporal studies of infected HeLa cells have indicated that virus is not detectable at nine hours post infection, but is demonstrable at 12 hours, mainly in the cytoplasm. By sampling at 26 hours post infection, Stoker et al. (1958) noted a large increase of particles within both the cytoplasm and nucleus.

Examination of human amnion cultures infected with the JM strain of HSV disclosed a number of nuclei containing aggregates of single membraned particles, which Morgan et al. (1958) termed "crystals." These aggregates contained, in addition to the usual single membraned particles, a number of particles devoid of nucleoids (electron dense cores). Inasmuch as aggregate or "crystal" formation could not be demonstrated with a different strain of virus, it was postulated that crystallization appeared to be a property of the strain of virus, rather than a state imposed by the host cell. Crystallization could be attributed to a rapid differentiation of virus within a loci, which suggested to Morgan and his group that viruses which crystallize during development would be less prone to reveal different developmental stages.

A study of fine structure of the mature particle was reported by Epstein (1962). The particle according to him, contains an electron-dense nucleoid, rod-shaped with round ends, lying in an inner zone of low density. Surrounding this is a dense outer zone or viroplasm without a membrane between the two zones. Permanganate fixation revealed a triple membraned particle, with the outer membrane morphologically resembling the plasma membrane.

Aggregates of intranuclear granules (approximately 20 mu in diameter), differing in size and electron density from marginated

chromatin, are associated with the development of this virus (Morgan et al., 1959). Based upon the observation that crystals of virus extend into these granules, it was considered that the intranuclear granules may be instrumental in synthesis of virus.

The manner in which virus is released from the nucleus and cytoplasm is virtually unknown. It has been suggested (Morgan et al., 1959) that reduplication of nuclear membranes occurs, inasmuch as the nuclear membranes remain essentially intact and tubules containing virus, indicative of budding through finger-like projections from the nucleus, have not been encountered. Cytochemical studies have indicated presence of ATPase activity on the microvilli of plasma membranes of HeLa cells and in the outer membranes of HSV particles grown in HeLa cells (Epstein and Holt, 1963). Cytoplasmic particles appear to escape from cells either by bulging as buds through cell membranes or through cytoplasmic vacuoles (Epstein, 1962).

Negative staining (Brenner and Horne, 1959) of free HSV particles with phosphotungstic acid has indicated that the virus consists of a polyhedral core with an average diameter of 77.5 mu (Wildy et al., 1960). The capsid (symmetrical shell), with an average diameter of 105.5 mu, consists of 162 substructures (capsomeres) with 5:3:2: symmetry. Capsomeres are hollow elongated hexagonal prisms with a mean diameter of 9.5 x 12.5 mu and a hole 4 mu in diameter extending through the middle. The outer membrane, or envelope, has a diameter of 180 mu.

In an attempt to determine whether inactivation of virus can be detected by morphological changes, Wildy and Watson (1962) determined particle: infectivity ratios of herpes virus grown in HeLa or

BHK 21 cells. From several different samples a high ratio of 24:1 and a low of 8:1 were obtained. Morphological differentiation of the 24:1 sample revealed 18 per cent enveloped particles (13 per cent full and 5 per cent empty) and 82 per cent naked particles (56 per cent full and 26 per cent empty), and the sample with a particle infectivity ratio of 8:1 revealed 16 per cent enveloped particles (12 per cent full and 4 per cent empty) and 84 per cent naked particles (63 per cent full and 21 per cent empty). Dialysis of one sample against either 1.8 per cent ammonium acetate or distilled water reduced infectivity by a factor of 2.5 without significantly altering the particle count.

CHAPTER III

MATERIALS AND METHODS

Tissue Culture Media

Two synthetic media[1], minimum essential medium (MEM) (Eagle, 1959) and Medium 199 (Morgan et al., 1950), were utilized for these studies. A modification of minimum essential medium (SMEM), in which $CaCl_2$ was omitted and $NaH_2PO_4 12H_2O$ was increased tenfold (Eagle, 1959), was employed in suspension culture studies.

Tissue Culture Media Supplements

Antibiotics

Stock solutions of penicillin[2] (100,000 units per ml) and streptomycin sulfate[3] (100,000 mcg per ml) were prepared in medium 199 or MEM, stored at -25° C, and diluted at time of usage to a final concentration of 100 mcg streptomycin sulfate and 100 units penicillin per ml media.

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[1]Microbiological Associates, Inc., Bethesda, Maryland.

[2]Parke, Davis and Company, Detroit, Michigan.

[3]E. R. Squibb and Sons, New York, New York.

Phenol Red

Phenol red (phenosulfonpthalein) was either present in trypsin or commercial media or, if not, added to a final concentration of 10 mg per liter.

Glutamine

Stock concentrates of glutamine[1] (200 mM) were stored at -25° C and added to MEM or SMEM at time of usage to a final concentration of 2 mM.

Calf Serum

All media were supplemented with calf serum[1] which was added at the time of usage. Inasmuch as supplementation of a chemically defined medium with a biological substance, such as serum, results in a chemically undefined medium, serum from the same lot was used in each experimental procedure where quantitative comparisons were made with the controls. Growth media, designated as G-MEM, SMEM, and G-199, were supplemented with ten per cent (volume/volume) calf serum.

Somatotropin

Beef somatotropin[4] (Raben type, 0.5 USP growth hormone units per ml) and human somatotropin[5] (quantitative biological activity unknown) were weighed on a Mettler micro balance, put into solution with a small volume of 0.01 N NaOH, quantitatively transferred to a volumetric

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[4]Nutritional Biochemicals Corporation, Cleveland, Ohio.

[5]Dr. D. K. Keele, Denton State School, Denton, Texas

flask, and brought to volume with MEM or media 199. Solutions were filtered through a bacteriological filter and stored as frozen stocks at -25° C. Somatotropin or somatotropin in combination with insulin or chorionic gonadotropin was diluted in media from the same lot utilized for proliferation of respective control cultures. Hormones were added to replicate tube cultures or suspension cultures at time of cell seeding. In some cases, cells were grown in the presence of hormone prior to seeding of cells into the experimental cultures.

p-Fluorophenylalanine

A solution of p-fluorophenylalanine[6] (FPA) was prepared at a concentration of 1 mM in SMEM and sterile-filtered at time of usage. Media used for dilution of FPA and control cultures for any particular experiment were from the same lot. This compound was incorporated into experimental cultures at various time intervals with respect to time of infection with virus. A final concentration of 0.5 mM FPA was utilized in all experiments.

Anti-human Growth Hormone (AHGH)

Anti-human growth hormone, prepared in rabbits, was obtained through courtesy of Dr. D. K. Keele, Department of Pediatrics of this institution. For studies in which AHGH (titer of 1:64) was utilized 0.5 ml of the antibody was mixed with somatotropin contained in 0.4 ml media and incubated at 37° C for one hour. Subsequently, the mixture was transferred quantitatively to suspension cultures.

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[6]Nutritional Biochemicals Corporation, Cleveland, Ohio.

Insulin

Iletin[7] (40 units zinc insulin per c.c.) was diluted in medium 199 at time of usage. This hormone alone or in combination with somatotropin was added to replicate tube cultures at time of cell seeding.

Chorionic Gonadotropin

Antuitrin-S[8] (5000 units per vial) was diluted in medium 199 at time of usage. This hormone alone or in combination with somatotropin was added to replicate tube cultures at time of cell seeding.

Trypsin

Stock solutions of trypsin (Difco 1:250)[9] of 2.5 per cent concentration (weight/volume) were prepared in calcium and magnesium free saline (GKN). Solutions were centrifuged at 500 g for 30 minutes at 5° C, sterilized by filtration, dispensed in ten ml portions, and stored at -25° C. A working solution was prepared by diluting a stock solution 1:10 with sterile GKN and adjusting pH to 7.6 with sterile 1.4 per cent $NaHCO_3$ (approximately six ml per 100 ml solution). GKN was prepared as a 10x stock solution by dissolving 80 gm NaCl, 4 gm KCl, and 10 gm glucose in one liter distilled water. Stock solution was stored at 5° C, and two ml chloroform per liter of stock was added as preservative. Working solutions of GKN were prepared by diluting 10x stock 1:10 with distilled water and autoclaving at 120° C for 15 minutes.

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[7] Eli Lilly and Company, Indianapolis, Indiana.

[8] Parke, Davis & Company, Detroit, Michigan.

[9] Difco Laboratories Incorporated, Detroit, Michigan.

Maintenance of Stock Tissue Culture

HeLa[10] (Gey et al., 1952) or FL human amnion[10] (Fogh and Lund, 1957) cells were grown in 160 ml Kimax milk dilution bottles. When cells appeared to be completely monolayered, generally at five to seven days post seeding, media were aseptically decanted and ten ml of 0.25 per cent trypsin were added and the cultures then were incubated at 37° C for 10 to 15 minutes. The resulting suspension of cells was removed, mixed with a pipette, and centrifuged lightly (300 g) for two to three minutes. G-MEM or G-199 was added to the sedimented cells in sufficient quantities (generally five to seven ml) for the resultant dilution to contain approximately 10^6 viable cells per ml. To each bottle containing ten to twelve ml of growth medium 10^6 viable cells were added. The bottles were allowed to incubate at 37° C in a horizontal position with media being changed every three or four days.

For some experiments it was evident that accurate measurement of biochemical changes should be noted on a per cell rather than per population basis. The submerged or spinner culture (McLimans et al., 1957, and Davis et al., 1958), which permits accurately reproducible sampling and enumeration of both cells and virus from the same population, was used in many of the studies. Due to temperature regulatory problems with spinner culture systems in closed incubators, it was found advantageous to use water jacketed flasks.[11] Water, pre-heated in a constant temperature water bath to 37° C, was circulated by a 1P501

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[10] Microbiological Associates, Inc., Bethesda, Maryland.

[11] Bellco Glass Co., Vineland, New Jersey.

Teel pump[12] in series through the flasks at a rate of approximately two liters per minute. Although methyl cellulose has been used in spinner cultures for a cushioning effect on cells (Bryant et al., 1960), it appeared to have little protective value on these cells and, due to the collodial nature of this substance, induced errors in enumerations of cells. Cultures were continuously gassed with five per cent carbon dioxide and 95 per cent air at a rate of approximately one liter per minute.

Stock Virus

The HF strain of herpes simplex virus (HSV), obtained through the courtesy of Dr. L. V. Scott, was utilized throughout this investigation. Homogeneity of stock virus was confirmed by Dr. P. A. Brunell (Virus Reference Unit, Communicable Disease Center, Atlanta, Georgia).

Virus was passed three times in HeLa cells and the fourth passage of virus was pooled and assayed for infectivity. This pooled stock (He-HF), containing $10^{6.0}$ $TCID_{50}$ per 0.1 ml, was stored in 1.0 ml portions in screw cap tubes at -65° C and employed in HeLa cell experiments. Assay of stock at termination of experiments which employed HeLa cells revealed a loss of $10^{0.3}$ $TCID_{50}$ per 0.1 ml.

The virus was passed an additional three times in FL amnion cells. Fourth passage of virus (HF-FL), pooled and utilized in all FL amnion cell experiments, contained $10^{6.5}$ $TCID_{50}$ per 0.1 ml.

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[12]Dayton Manufacturing Company, Chicago, Illinois.

Electron Microscopy[13]

Samples of cells for electron microscopy studies were placed in small (two ml) conical tubes, centrifuged (300 g), decanted, and resuspended in 0.25 per cent trypsin for ten minutes at 37° C to remove cellular debris. Subsequently, cells were centrifuged (300 g), fixed in Palade's buffered fixative (one per cent osmium tetroxide in 0.14 M sodium veronal-acetate adjusted to pH 7.4 with 0.1 n HDl) for 0.5 hours at 4° C, and dehydrated in graded series (15 minutes in each at 4° C) of ethanol (50, 70, 95, and 100 per cent). Tissues were infiltrated with propylene oxide (two changes of 15 minutes each) and propylene oxide-Maraglas mixture[14] (68 per cent Maraglas 655; 20 per cent Cardolite NC 513; ten per cent dibutylphthalate; and two per cent benzyldimethylamine), 1:1 for 30 minutes and finally in Maraglas mixture overnight. After this they were embedded in gelatin capsules and polymerized at 60° C for 48 hours. Sections were cut on a Porter-Blum Model I ultra microtome[15] and those having silver interference colors, indicating 60 to 90 mu thickness, were mounted on carbon coated 200 mesh grids and examined with an RCA EMU-3F electron microscope. In some instances, sections were stained with saturated uranyl acetate for two hours prior to microscopic examination.

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[13]With the technical assistance of Mr. Randy Scott, University of Oklahoma Medical Center, Oklahoma City, Oklahoma.

[14]Polysciences, Incorporated, Rydal, Pennsylvania.

[15]Ivan Sorvall, Incorporated, Norwalk, Connecticut.

Quantitative Methods

Titration of Infectious Virus

HeLa or FL human amnion cells were cultivated as previously described (Maintenance of Stock Tissue Culture). Suspensions of cells (three to five x 10^5 viable cells per ml growth medium) were distributed in 0.5 ml portions into rubber stoppered tubes with a Cornwall continuous pipettor. Tubes were incubated in stationary racks at 37° C until cells appeared to about 90 per cent monolayered (generally in three to four days), at which time they were used for assay of virus.

Cell samples for infectious virus titrations, which had been stored at -65° C (usually no more than three to four weeks prior to assay), were quickly frozen and thawed three times (acetone at -25° C and water at 37° C) at the time of virus assay to release as much cell-associated virus as possible. The resulting virus suspensions were prepared in serial 10-fold dilutions in maintenance medium at 4° C, with separate 0.2 ml pipettes being used for each transfer. Growth medium was removed from replicate tube cultures and four to six tubes were inoculated with 0.1 ml of each virus dilution. Virus was allowed to adsorb for one hour at 37° C, after which 0.5 ml maintenance medium was added to each tube. The rubber stoppered tubes were incubated at 37° C in a stationary position and examined three and five days after infection for characteristic cytopathic effects (plaques with giant cells). Tubes, examined microscopically, were recorded as positive or negative with respect to cytopathic effects and the tissue culture infectious dose fifty per cent ($TCID_{50}$) was calculated by the method of Reed and Muench

(1938). The possibility of introducing any effect of FPA from virus suspensions replicated in presence of FPA into the determinations of infectious virus, was minimized by increasing phenylalanine in maintenance media from 0.2 mM to 1.0 mM.

Determination of Protein

In several experiments the content of cellular protein was determined. The Oyama and Eagle (1956) modification of the Lowry method for total protein determination (Lowry et al., 1951) was utilized. Fifty parts of reagent A (20 gms Na_2CO_3, 4 gms NaOH, and 0.2 gms Na-K tartrate per liter distilled water) were mixed with one part of reagent B (0.5 gms $CuSO_4.5H_2O$ per 100 ml distilled water) to form reagent C which was freshly prepared for each set of determinations. Working standards (10 to 100 mcg protein per ml distilled water) were freshly prepared for each set of determinations from a sterile stock standard of Armour[16] bovine serum albumin, containing 10.5 mg protein nitrogen per ml. Suspensions of cells were washed twice with Hank's balanced salts solution (HBSS), centrifuged (300 g), inverted and allowed to drain for 15 minutes after which 1.0 ml distilled water was added. Five ml reagent C were added to 1.0 ml of the working standards or unknowns and allowed to incubate at room temperature for 30 minutes. Five-tenths ml Folin-Ciocalteau reagent[17] was added to the mixture (diluted 1:2 with distilled water at time of usage) and incubated at room temperature for an additional 60 minutes. Results were read at 650 mμ in a Bausch and Lomb Model 340 spectrophotometer using a reagent-distilled water

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[16] Armour Pharmaceutical Company, Kankakee, Illinois.

[17] Fisher Scientific Company, Fair Lawn, New Jersey.

blank for zero absorbancy. Standards were plotted on semilog graph paper and the concentrations of protein, expressed in terms of bovine serum albumin, were determined by reference to the standard curves.

Determination of Glucose

The amounts of glucose present in supernatant media were determined in some experiments. Cell suspensions from the spinner cultures were centrifuged lightly (300 g) and supernatant media were removed. Appropriate dilutions of "unknown" were made with phosphate buffered saline (pH 7.0) so that "unknown" contained between 50 and 300 mcg D-glucose per ml.

Glucostat[18], a coupled enzyme system, was utilized for quantitative colorimetric determinations of D-glucose. The principle of the determination is based on the following scheme of reactions (Beach and Turner, 1958):

(1) $\text{Glucose} + O_2 + H_2O \xrightarrow[\text{glucose oxidase}]{} H_2O_2 + \text{gluconic acid}$

(2) $H_2O_2 + \text{reduced chromogen} \xrightarrow[\text{peroxidase}]{} \text{oxidized chromogen}$

Working standards of 50 to 300 mcg D-glucose were prepared from a stock standard of reagent grade D-glucose[19] (10 mg/ml saturated benzoic acid). Lyophilized enzymes and chromogen were reconstituted for each set of determinations according to directions supplied with the reagents. One ml diluted unknown, standard, or water was added to 9.0 ml reagent. All samples were incubated at room temperature for ten minutes and then the reaction was stopped by adding one drop of 4.0 M HCl. Color was stabilized for five minutes and measured in a Bausch

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[18]Worthington Biochemical Corporation, Freehold, New Jersey.

[19]Fisher Scientific Company, Fair Lawn, New Jersey.

and Lomb Model 340 spectrophotometer at a wave length of 400 mμ, using the reagent blank for zero absorbancy. Standards were plotted on semi-log graph paper and concentrations of unknowns, expressed in terms of D-glucose, were established from the standard curves.

Enumeration of Cells

At various times after incubation at 37°C, media from replicate tube cultures of hormone treated cells and control cells were decanted. Resulting layers of cells were washed twice to remove cellular debris by gently pipetting two ml 1x GKN into the inner surface of the tube, gently rotating the liquid over the surface of the cells, and subsequently decanting the liquid. To the resulting monolayers of cells two ml of a 0.25 per cent solution of trypsin was added and allowed to incubate at 37°C for 10 to 15 minutes for enumeration of the resulting cell suspension.

Initial studies utilized the Coulter electronic counter[20] for enumeration of the resulting cell suspensions. Attempts were made to count whole cells with the apparatus, but this proved to be unsatisfactory because of gross clumping of cells. Lysing of cells with 0.1 M citric acid and counting only nuclei overcame this difficulty. However, in order to screen out interference created by cellular debris, it became necessary to adjust the lower threshold to a reading of 12. After cells were lysed, filtered saline was added to adjust populations to between 5,000 and 20,000 per 0.5 ml. A minimum of three counts was made on each sample with the controls of the instrument at the following

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[20]Coulter Electronics, Chicago, Illinois.

settings: amplification-2, aperture-$\frac{1}{2}$, lower threshold-12, and upper threshold-100. Parallel counting with the electronic counter and hemocytometer indicated that direct hemocytometer enumerations were approximately five per cent less than those counted with the instrument. The average (arithmetical mean), from which no count deviated more than two per cent, was corrected for background interference and for multiplicity of particles entering the chamber simultaneously. Direct hemocytometer counts of nuclei were made by diluting cell suspensions with 0.1 per cent crystal violet in 0.1 M citric acid (Sanford et al., 1951). When it was deemed necessary to determine viable cell populations (from suspension cultures), suspensions of cells were diluted 1:3 with 0.1 per cent trypan blue (Davis et al.,1958) without prior treatment with trypsin.

CHAPTER IV

RESULTS

Effects of Hormone on Replicate Tube Cultures of HeLa Cells

Beef Growth Hormone

Table 1 illustrates results of a typical experiment in which beef growth hormone (BGH), diluted in G 199, was added in varying concentrations to replicate tube cultures of HeLa cells at time of cell seeding. Tubes were incubated at 37°C for 72 hours. Enumeration of cells, expressed as total cell population per tube, showed that 0.005 mcg BGH per ml of medium induced the greatest proliferation of cells. Higher concentrations of hormone appeared not to be stimulatory, but the cell populations approached those of control cultures.

Table 2 shows results of an experiment in which 2.5 mcg BGH per ml of G 199 was added at time of seeding of cells and populations of cells were examined as a function of time. Content of calf sera in media was reduced to 5 per cent (volume/volume). Media were changed on remaining replicate tubes 69 hours post-seeding. Cells not in the presence of hormone attained approximately the same population density as those with hormone, but those in the presence of hormone appeared to be accelerated to a faster growth rate.

TABLE 1

EFFECTS OF VARIOUS CONCENTRATIONS OF BEEF GROWTH HORMONE ON PROLIFERATION OF HELA CELLS 72 HOURS POST-SEEDING

Concentration of Hormone[a]	Cell Count	Percentage of Control
50	2.12[b]	88
5	2.22	91
0.5	2.26	92
0.05	2.54	104
0.005	3.18	130
0.0005	3.02	124
0 (control)	2.44	-

[a] Expressed as mcg beef growth hormone per ml of medium 199 supplemented with 10 per cent calf serum.

[b] Total cell population per tube x 10^{-5}. Tubes seeded at 0 time with 1.56 x 10^{5} cells. Average of duplicate experiment.

TABLE 2

EFFECT OF 2.5 MCG BEEF GROWTH HORMONE PER ML MEDIUM[a] ON PROLIFERATION OF HELA CELLS

Hours Post-Seeding	Total Cell Populations: 2.5 mcg BGH[b]	Total Cell Populations: Control Medium Without Hormone	Percentage of Control[c]
0	1.71[d]	1.71	
18	2.20	2.24	98.2
42	4.38	3.84	114.0
68	5.15	4.26	120.8
69	Medium Changed	Medium Changed	
114	6.01	5.79	103.7
139	5.34	5.27	101.3
163	4.41	5.68	77.6

[a]Medium 199 supplemented with 5 per cent calf serum.

[b]Beef growth hormone per ml medium.

[c]Calculated on basis of cell population with hormone/ cell population without hormone x 100.

[d]Total cell population per tube x 10^{-5}. Average of triplicate experiment.

Inasmuch as serum contains hormones (but is required as a supplement for growth of tissue culture cells), it was possible that the stimulatory effects of growth hormone might be modified with respect to concentration of serum. To test this possibility, cells were subcultured for 4 days in: (1) G 199 supplemented with 10 per cent calf serum; (2) G 199 supplemented with 10 per cent calf serum and 2.5 mcg BGH per ml medium; (3) G 199 supplemented with 5 per cent calf serum, and (4) G 199 supplemented with 5 per cent calf serum and 2.5 mcg BGH per ml medium. Monolayers of cells were trypsinized and replicate tube cultures of each, containing homologous medium, were initiated. Results, as shown in Table 3, indicated that approximately the same maximum yield of cells is attained under influence of the four different environmental conditions, but those under influence of growth hormone or increased concentration of serum have decreased generation times.

It was of interest to determine whether or not protein synthesis was stimulated by the hormones to the same extent as cellular proliferation. Replicate tube cultures were incubated in the presence of 5.0 mcg BGH per ml medium and without hormone. After cells had incubated 72 and 120 hours, total content of protein and total cell population per culture were determined. Results of this experiment, as shown in Table 4, indicated that populations of cells under influence of hormone were greater than those without hormone and total protein of cell population was slightly less than that of controls. Conversion of total protein per population to total protein per cell indicated that cells under influence of BGH contained 23 per cent less protein per cell than did control cells (72 hours post-seeding). A similar comparison

TABLE 3

EFFECTS ON PROLIFERATION OF HELA CELLS OF VARYING CONCENTRATIONS OF SERUM AND 2.5 MCG BEEF GROWTH HORMONE PER ML MEDIUM

Hours Post-Seeding	199_{10}[a]			199_{5}[b]		
	2.5 mcg BGH[c]	Control[d]	Percentage of Control[e]	2.5 mcg BGH	Control	Percentage of Control
0	1.47[f]	1.47		1.38	1.38	
51	3.15	2.56	123	2.61	2.48	105
70	4.28	4.05	106	3.92	2.75	143
76	4.87	4.23	115	4.38	3.22	136
77	Medium Changed			Medium Changed		
96	7.10	6.40	111	6.51	5.09	128
123	9.64	8.69	111	8.89	5.57	160
168	8.77	9.04	97	8.83	7.84	113

[a] Medium 199 supplemented with 10 per cent (volume/volume) calf serum.

[b] Medium 199 supplemented with 5 per cent (volume/volume) calf serum.

[c] 2.5 gamma beef growth hormone per ml medium.

[d] Control medium without hormone.

[e] Calculated on basis of cell population with hormone/ cell population without hormone x 100.

[f] Total cell population per tube x 10^{-5}. Average of duplicate experiment.

TABLE 4

EFFECTS ON POPULATION OF HELA CELLS AND CONTENT OF CELLULAR PROTEIN OF 5.0 MCG BEEF GROWTH HORMONE PER ML MEDIUM 199

Hours Post-Seeding	Medium with Hormone				Medium with Hormone		
	CP[a]	P[b]	PC/C[c]	CP%C[d]	CP[a]	P[b]	PC/C[c]
72	5.82	104.7	18.0	136.2	4.28	110.0	23.4
120	4.98	102.3	20.6	101.0	4.94	116.2	23.5

[a]Total cell population per tube x 10^{-5}. Average of quadruplet experiment.

[b]Total content of protein (mcg) per tube. Average of quadruplet experiment.

[c]Protein content expressed as mcg protein per cell x 10^{5}.

[d]Cell population percentage of replicate controls. Calculated on basis of cell population with hormone/cell population without hormone x 100.

of cells 120 hours after seeding indicated that those under hormonal influence contained 12 per cent less protein than did control cultures.

Insulin

Results of an experiment in which replicate tube cultures of HeLa cells were incubated in the presence of various concentrations of insulin are shown in Table 5. Enumeration of cells 72 hours post-seeding indicated that insulin, in the various concentrations tested, was slightly to markedly inhibitory to cellular replication.

Chorionic Gonadotropin

Table 6 illustrates the results of an experiment in which replicate tube cultures were seeded at 0 time with equal numbers of cells. One set of tubes contained 5 units of chorionic gonadotropin per ml medium (G 199) supplemented with 5 per cent calf serum. A set of replicate tube cultures, without hormone, served as control cultures. Results suggested that this hormone had little effect on cellular proliferation, but cells in presence of the hormone appeared in death phase earlier.

Effect of Various Combinations of Hormones on Replicate Cultures of HeLa Cells

Growth Hormone and Insulin

Results of an experiment in which 10 mcg growth hormone plus varying amounts of insulin (2, 1, or 0.1 units per ml G 199) are shown in Table 7. Results of this experiment indicated that a combination of BGH and insulin was stimulatory to cellular proliferation, especially with the lower concentrations of insulin.

TABLE 5

EFFECTS ON PROLIFERATION OF HELA CELLS OF VARIOUS CONCENTRATIONS OF INSULIN

Concentration of Hormone[a]	Cell Count[b]	Percentage of Control Without Hormone[c]
5.0	3.04	82
0.5	3.30	89
0.05	3.26	88
0.005	3.38	91
0.0005	2.66	72
0	3.72	-

[a] Units insulin per ml medium 199 supplemented 10 per cent calf serum.

[b] Total cell population per tube x 10^{-5}. Average of duplicate experiment. Tubes seeded at 0 time with 1.56×10^{5} cells and enumerated 72 hours after seeding.

[c] Calculated on basis of cell population with hormone/cell population without hormone x 100.

TABLE 6

EFFECTS ON PROLIFERATION OF HELA CELLS OF 5 UNITS CHORIONIC GONADOTROPIN PER ML MEDIUM 199

	Total Cell Populations		
Hours Post-Seeding	Medium with Hormone[a]	Control Medium without Hormone[b]	Percentage of Control[c]
24	1.48[d]	1.50	98.6
47	2.76	2.61	105.7
71	3.79	4.08	92.8
95	2.93	4.03	72.7

[a]Medium supplemented with 5 per cent calf serum and 5 units chorionic gonadotropin.

[b]Medium supplemented with 5 per cent calf serum.

[c]Calculated on basis of cell population with hormone/cell population without hormone x 100.

[d]Total cell population per tube x 10^{-5}. Average of 5 duplicate experiment. Tubes seeded with 1.63 x 10^{5} at 0 time.

TABLE 7

EFFECTS ON PROLIFERATION OF HELA CELLS OF 10.0 MCG BEEF GROWTH HORMONE AND VARYING AMOUNTS OF INSULIN PER ML MEDIUM 199

Hours Post-Seeding	Units Insulin per ml Medium							
	0[a]		2		1.0		0.1	
	CP[b]	CP%C[c]	CP	CP%C	CP	CP%C	CP	CP%C
24	3.00	-	2.62	87	2.80	93	2.84	95
48	4.68	-	4.56	98	5.24	112	4.74	101
49			Medium Changed					
72	7.72	-	8.12	105	8.56	111	9.48	123
96	9.92	-	8.18	83	9.64	97	10.40	105

[a]Control cultures with BGH (10 mcg per ml medium 199 supplemented with 10 per cent calf serum). Other cultures contained 10.0 mcg BGH per ml medium plus indicated units of insulin per ml medium.

[b]Total cell population per tube x 10^{-5}. Average of duplicate experiment. Tubes seeded with 2.98 x 10^{5} cells at 0 time.

[c]Cell population percentage of control. Calculated on basis of cell population with insulin/cell population without insulin x 100.

In a similar experiment the influence of 5.0 mcg BGH and 0.5 unit insulin per ml G 199 on proliferation and protein content of HeLa cells were determined. As shown in Table 8, combinations of BGH and insulin, in the amounts indicated, were stimulatory on cellular proliferation, but protein content of population appeared to be similar. However, determinations of protein content, expressed on a per cell basis, revealed that cells incubated in presence of the hormones contained less cellular protein than did controls.

Growth Hormone and Chorionic Gonadotropin

Incubation of replicate tube cultures with varying amounts of chorionic gonadotropin and BGH was inhibitory to cellular proliferation in the amounts tested (Table 9). These inhibitory effects increased with increasing concentrations of hormone and longer time of incubation.

Effect of Various Hormones on Replication of Herpes Simplex Virus in HeLa Cells

Monolayers of HeLa cells were incubated in presence of various hormones, as indicated in Table 10, for a period of 18 hours prior to infection. Media were removed from each bottle and 2×10^4 $TCID_{50}$ (tissue culture infective dose 50 per cent) HSV was added to each bottle. Virus was allowed to adsorb for one hour at $37^{\circ}C$, at which time inoculum was removed and G 199 supplemented with 5 per cent calf serum and homologous hormone was added to each bottle (final volume of 12 ml per bottle). At times indicated 1.0 ml of media was removed from each bottle and replenished with 1.0 ml homologous medium. Titration of samples indicated that HGH, in concentrations of 5 to 10 mcg per ml of media, reduced yield of virus during early stages of infection, but a

TABLE 8

EFFECTS OF PROLIFERATION OF HELA CELLS AND CONTENT OF CELLULAR PROTEIN OF 5.0 MCG BEEF GROWTH HORMONE AND 0.5 UNIT INSULIN PER ML MEDIUM 199

Hours Post-Seeding	Medium with Hormones				Medium without Hormones		
	CP[a]	P[b]	PC/C[c]	CP%C[d]	CP	P	PC/C
48	3.96	85	21.4	123.7	3.20	100	31.2
72	5.22	100	19.1	144.2	3.62	105	29.0
120	3.92	90	22.9	110.7	3.54	110	31.1

[a] Total cell population per tube x 10^{-5}. Average of quadruplet experiment.

[b] Total content of protein (mcg) per tube. Average of quadruplet experiment.

[c] Protein content expressed as mcg protein per cell x 10^{5}.

[d] Cell population per cent of replicate controls. Calculated on basis of cell population with hormone/cell population without hormone x 100.

TABLE 9

EFFECTS ON PROLIFERATION OF HELA CELLS OF 2.5 MCG BEEF GROWTH HORMONE AND VARYING AMOUNTS OF CHORIONIC GONADOTROPIN PER ML OF MEDIUM 199

Hours Post-Seeding	Units Chorionic Gonadotropin							
	0[a]		2.5		5.0		10.0	
	CP[b]	CP%C[c]	CP	CP%C	CP	CP%C	CP	CP%C
23	1.38	-	1.04	75	1.24	90	1.26	91
48			Medium Changed					
75	6.74	-	5.86	87	5.02	74	3.26	48
93	7.94	-	6.84	86	5.48	69	2.58	32

[a] Control cultures with 2.5 mcg beef growth hormone per ml medium 199 supplemented with 5 per cent calf serum. Other cultures contained 2.5 mcg beef growth hormone plus the indicated units of chorionic gonadotropin per ml medium.

[b] Total cell population per tube x 10^{-5}. Tubes seeded with 2.22 x 10^5 cells at 0 time.

[c] Cell population per cent of replicate controls without chorionic gonadotropin. Calculated on basis of cell population with CGT/cell population without CGT x 100.

TABLE 10

EFFECTS OF VARIOUS HORMONES[a] ON REPLICATION OF HERPES SIMPLEX VIRUS IN HELA CELLS

Hours Post-Infection[b]	Titers of Virus					
	5 mcg HGH[c]	10 mcg HGH	5 u CGT[d]	10 u CGT	1 u Insulin[e]	Control
4	2.00[f]	1.75	1.75	2.60	2.75	2.75
7	2.23	2.33	2.00	2.50	2.50	2.33
24	1.50	1.66	1.50	1.50	2.53	6.33
31	2.83	3.66	2.66	2.50	<1.00	6.50
49	6.50	6.50	4.33	4.75	3.30	6.00
56	6.50	6.25	6.00	6.24	1.43	6.50

[a]Added 18 hours prior to infection.

[b]Monolayer cultures infected with 2 x 10^4 $TCID_{50}$ HSV per bottle.

[c]Human growth hormone per ml MEM supplemented with 5 per cent calf serum (volume/volume).

[d]Units chorionic gonadotropin per ml MEM supplemented with 5 per cent calf serum (volume/volume).

[e]Units insulin per ml MEM supplemented with 5 per cent calf serum (volume/volume).

[f]Virus titer expressed as positive $\log_{10}$ $TCID_{50}$ per ml medium.

comparable amount of virus was produced during later stages of infection. A comparison of infective virus present in hormone treated cells with non-treated cells four hours post-infection, probably residual virus inoculum, indicated less virus in supernatant media or CGT and HGH treated cells. Cells in presence of five to ten units CGT exhibited an effect similar to that induced by HGH. Insulin reduced final yield of virus. Cytopathic effect of virus in control cultures was characterized by formation of well-defined macroplaques and polykaryocytes. Visual cytopathic changes of cells in presence of HGH or CGT appeared similar to control cultures, but slower in developing cytopathic effects. Cells in presence of insulin, however, did not exhibit formation of polykaryocytes and gross observations indicated plaques to be smaller and fewer in number.

Effects of Human Growth Hormone (HGH) on Infected and Non-Infected FL Human Amnion Cells in Suspension Culture

Effects of HGH on FL Human Amnion Cells

Two spinner flasks, one containing 4.31 mcg HGH per ml SMEM and the other without hormone, were seeded with equal numbers of viable cells. At various times after seeding of cells viable populations and protein content of cells were determined. As shown in Table 11, cellular stimulation was apparent within 20 hours, with maximum stimulation occurring at approximately 42 hours post-seeding. Protein content per cell decreased in cells in the presence of hormone and, as shown in Table 12, this difference became greater with increasing length of time.

To determine whether or not a correlation existed between amount of HGH and response of cells, equal numbers of viable cells were seeded

TABLE 11

EFFECT OF 4.31 MCG HUMAN GROWTH HORMONE PER ML MEDIUM[a] ON PROLIFERATION OF FL AMNION CELLS IN SUSPENSION CULTURE

Hours Post-Seeding	Population of Viable Cells: 4.31 mcg HGH[b]	Population of Viable Cells: Control Medium without Hormone	Percentage of Control[c]
0	1.24[d]	1.24	100
20	3.00	2.32	129
42	3.80	2.16	176
66	5.16	3.60	143

[a] SMEM supplemented with 10 per cent calf serum.

[b] Human growth hormone per ml medium.

[c] Calculated on basis of cell population with hormone/cell population without hormone x 100.

[d] Population of viable cells per ml medium x 10^{-5}.

TABLE 12

EFFECTS OF 4.31 MCG HUMAN GROWTH HORMONE PER ML MEDIUM[a] ON THE PROTEIN CONTENT OF FL AMNION CELLS

Hours Post-Seeding	Medium with Hormone[b]			Medium without Hormone		
	Protein[c]	CP[d]	Protein per Cell[e]	Protein	CP	Protein per Cell
42	89	3.80	2.342	76	2.16	3.518
66	89	5.16	1.725	136	3.60	3.777

[a]SMEM supplemented with 10 per cent calf serum.

[b]4.31 mcg HGH per ml medium.

[c]Mcg protein per ml of cells.

[d]Population of viable cells per ml medium x 10^{-5}.

[e]Mcg protein per cell x 10^{4}.

into five spinner flasks which contained the following: (1) 0.1 mcg HGH per ml SMEM; (2) 1.0 mcg HGH per ml SMEM; (3) 10.0 mcg HGH per ml SMEM; (4) 10.0 mcg HGH per ml SMEM and 0.5 ml rabbit anti-HGH antibody (hormone and anti-HGH incubated together one hour at 37°C prior to addition to flask) and (5) an equivalent volume of normal rabbit sera (NRS) to that used in flask number 4. Table 13 indicates that increasing amounts of human growth hormone resulted in enhanced growth of FL amnion cells, but a linear response or log-dose response was not observed. The marked stimulatory response to 10 mcg HGH was inhibited by anti-HGH.

Electron microscopic examination of FL amnion cells in the presence of 4.31 mcg HGH per ml SMEM (Figure 1) and without hormone (Figure 2) indicated cells of normal morphology with well developed mitochondria (M). Mitotic figures (Figure 1) were repeatedly seen in cells in presence of HGH, but were rarely encountered in cells not incubated in presence of hormone when sampled at the same time (41 hours post-seeding). Gross observations suggested, by comparisons of Figures 1 and 2, the presence of considerably more abundant cytoplasm (C) in cells which were not grown in presence of hormone.

Effect of HGH on Glucose Utilization

Cells were grown in suspension cultures with and without HGH for 50 hours. Media were removed and two flasks, one containing 4.31 mcg HGH per ml SMEM and the other without HGH, were seeded with equal numbers of FL cells. Glucose present in media was determined in each flask at time of seeding and at 21, 42 and 47 hours post-seeding. Rate of glucose utilization, as shown in Table 14, was calculated by dividing amount of glucose utilized in the preceding time period by number of

TABLE 13

EFFECT OF HUMAN GROWTH HORMONE CONCENTRATION ON PROLIFERATION OF FL AMNION CELLS IN SUSPENSION CULTURE

Concentration of Hormone[a]	Population of Viable Cells 18 Hours after Seeding[b]
0.1	1.54[c]
1.0	2.54
10.0	2.94
10.0 + AHGH[d]	2.08
NRS[e]	2.30

[a] Expressed as mcg human growth hormone per ml medium.

[b] 1.48 viable cells per ml medium seeded at 0 time.

[c] Viable cells per ml medium x 10^{-5}.

[d] Rabbit anti-human growth hormone antibody incubated with human growth hormone 1 hour at 37°C before adding to medium.

[e] An equivalent volume of non-immune rabbit serum.

TABLE 14

EFFECT OF 4.31 MCG HUMAN GROWTH HORMONE ON RATE OF DEPLETION OF D GLUCOSE BY FL AMNION CELLS

Hours Post-Seeding	4.31 mcg HGH[a]			Control without Hormone		
	Rate of Glucose Depletion[b]	CP[c]	Rate of Depletion Per Cell[d]	Rate of Glucose Depletion	CP	Rate of Depletion Per Cell
0		1.60			0.84	
21	13.33	3.81	3.500	17.14	1.71	10.023
42	12.00	2.49	4.819	24.00	2.13	11.267
47	9.47	2.43	3.979	26.30	2.64	9.962

[a]Mcg human growth hormone per ml SMEM.

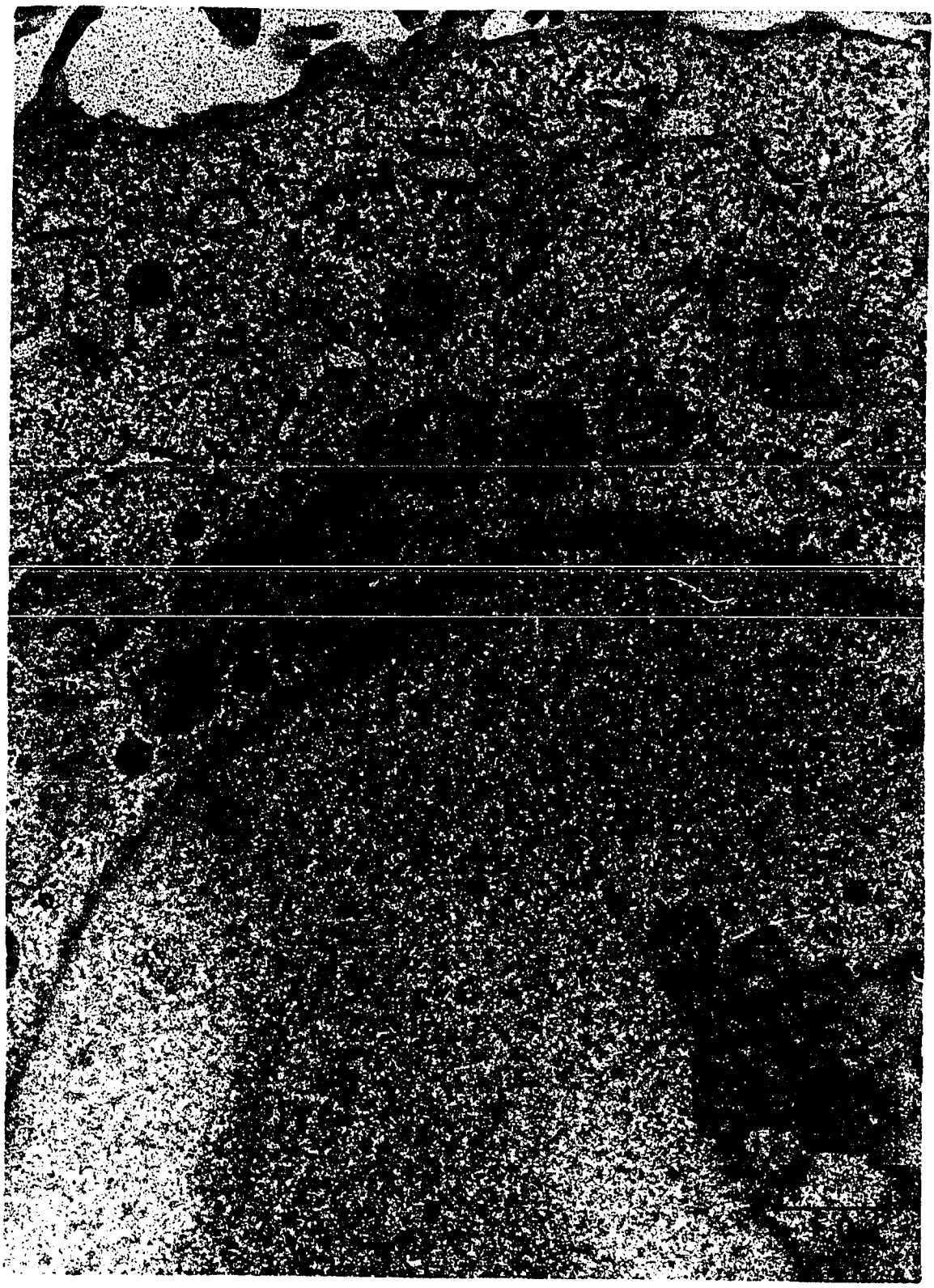
[b]Average rate of D glucose depletion expressed as mgs D glucose per hour per ml medium x 10^3.

[c]Viable cell population per ml medium x 10^{-5}.

[d]Average rate of D glucose depletion expressed as mgs D glucose per hour per cell x 10^8.

Figure 1. FL Human Amnion Cell Grown in Presence of 4.31 mcg Human Growth Hormone per ml Medium.

Figure 2. Normal FL Human Amnion Cell.



hours in that time period. Results indicated that the population of cells in presence of hormone were catabolizing considerably less glucose than those without hormone. Converting rate of glucose utilization to a per cell basis further points out this difference.

Effects of HGH on Replication of HSV

Two spinner cultures, one containing 4.31 mcg HGH per ml SMEM and the other without hormone, were seeded with equal numbers of cells. Fifty hours later respective media were changed, and cells were enumerated in each flask and infected with 0.4 $TCID_{50}$HSV per cell. At various times after infection (as shown in Table 15), samples were removed from each flask and the titers of infectious virus, expressed as $TCID_{50}$HSV per 0.1 ml cell-containing media, were determined. The first samples, 0.5 hour post-infection, showed slightly more infectious virus in the flask without hormone than in the flask which contained hormone. Analyses of other samples for infectious virus revealed that cells in presence of HGH produced comparable amounts of infectious virus to control cultures, but rate of synthesis was reduced.

To determine whether or not this response could be modified with respect to concentration of hormone, cells were grown under the influence of the five following different environmental conditions: (1) 0.1 mcg HGH per ml SMEM; (2) 1.0 mcg HGH per ml SMEM; (3) 10 mcg HGH per ml SMEM; (4) 10.0 mcg HGH per ml SMEM and 0.5 ml rabbit anti-HGH antibody (hormone and anti-HGH incubated together one hour at 37°C prior to addition to flask), and (5) equivalent volume of normal rabbit sera (NRS) to that used in flask number 4. After 18 hours incubation they were infected with 0.4 $TCID_{50}$HSV per cell. Results are shown in

TABLE 15

EFFECT OF 4.31 MCG HUMAN GROWTH HORMONE PER ML MEDIUM ON THE PROLIFERATION OF HERPES SIMPLEX VIRUS IN SUSPENSION CULTURE

Hours Post-Inoculation	Titers of Virus	
	4.31 HGH[a]	Control Without Hormone
0.5	2.10[b]	2.83
21.0	3.41	3.31
26.0	3.50	5.24
45.0	3.63	2.63
53.0	5.16	3.38
75.0	4.30	3.50

[a]Mcg human growth hormone per ml SMEM. Cells grown in presence of hormone for 50 hours. Media changed prior to infection with 0.4 $TCID_{50}$ HSV per cell.

[b]Titer of infectious virus expressed as positive $\log_{10}$ $TCID_{50}$ per 0.1 ml media.

Table 16 of virus titrations of samples removed 6.5, 24.5, and 29 hours post-infection. Infectious virus present 6.5 hours post-infection was slightly less in hormone treated cells. Increasing concentrations of hormone resulted in decreasing amounts of infectious virus produced at the times indicated. When HGH and anti-HGH antibody were incubated together prior to addition to this system, hormonal effects were nullified and virus titers approached those of the control culture which was supplemented with NRS.

Electron microscopic findings of infected FL amnion cells (40 hours post-infection), not pretreated with HGH, ranged from markedly necrotic cells exhibiting intranuclear Cowdry type A inclusion (A, Figure 3) which contained few, if any, virus particles to polykaryocytes (Figure 4), which contained double membraned particles (DMP) in the cytoplasm and single membraned particles (SMP) within the nucleus. Adjacent to nuclear membranes (NM) and marginated chromatin (MC) (in Figure 4) are several areas of medium sized granules, which for clarity will be termed "B granules" (BG). Focal accumulations of single membraned particles appear randomly dispersed throughout the remainder of the nuclear matrix.

Examination of FL cells infected in presence of 4.31 mcg HGH (Figures 5 and 6) revealed a similar pattern of development, but in several cells a number of unusual morphological forms, not previously encountered in virus infected controls or non-infected control cultures (with or without HGH), was observed. Figure 5 illustrates a markedly necrotic cell with extensive vacuolization of the cytoplasm. Cytopathic effect is also noted by margination of chromatin (MC) and a discontinuous

TABLE 16

EFFECTS OF CONCENTRATION OF HUMAN GROWTH HORMONE ON THE REPLICATION OF HERPES SIMPLEX VIRUS IN SUSPENSION CULTURES

Concentration of Hormone[a]	Hours Post-Infection		
	6.5	24.5	29.0
0.1	3.33[b]	4.66	4.50
1.0	3.33	4.00	4.29
10.0	3.33	3.50	3.83
10.0 + AHGH[c]	3.66	5.33	5.18
NRS[d]	3.66	4.72	5.37

[a] Expressed as mcg human growth hormone per ml SMEM. Cells grown in presence of hormone for 18 hours and subsequently infected with 0.4 $TCID_{50}$ HSV per cell.

[b] Titer of virus expressed as positive $\log_{10}$ $TCID_{50}$ per 0.1 ml medium.

[c] Rabbit anti-human growth hormone antibody incubated with human growth hormone 1 hour 37°C before adding to medium.

[d] Equivalent volume of non-immune rabbit serum.

Figure 3. FL Human Amnion Cell Infected with Herpes Simplex Virus Forty Hours Post-Infection. Intranuclear Cowdry Type A Inclusion.

Figure 4. FL Human Amnion Cell Infected with Herpes Simplex Virus. Forty Hours Post-Infection. Formation of Polykaryocyte.


MC
NM
SMP
BG
DMP

Figure 5. FL Human Amnion Cell Infected with Herpes Simplex Virus. Forty Hours Post-Infection. Cells Grown in Presence of 4.31 mcg Human Growth Hormone per ml Medium.

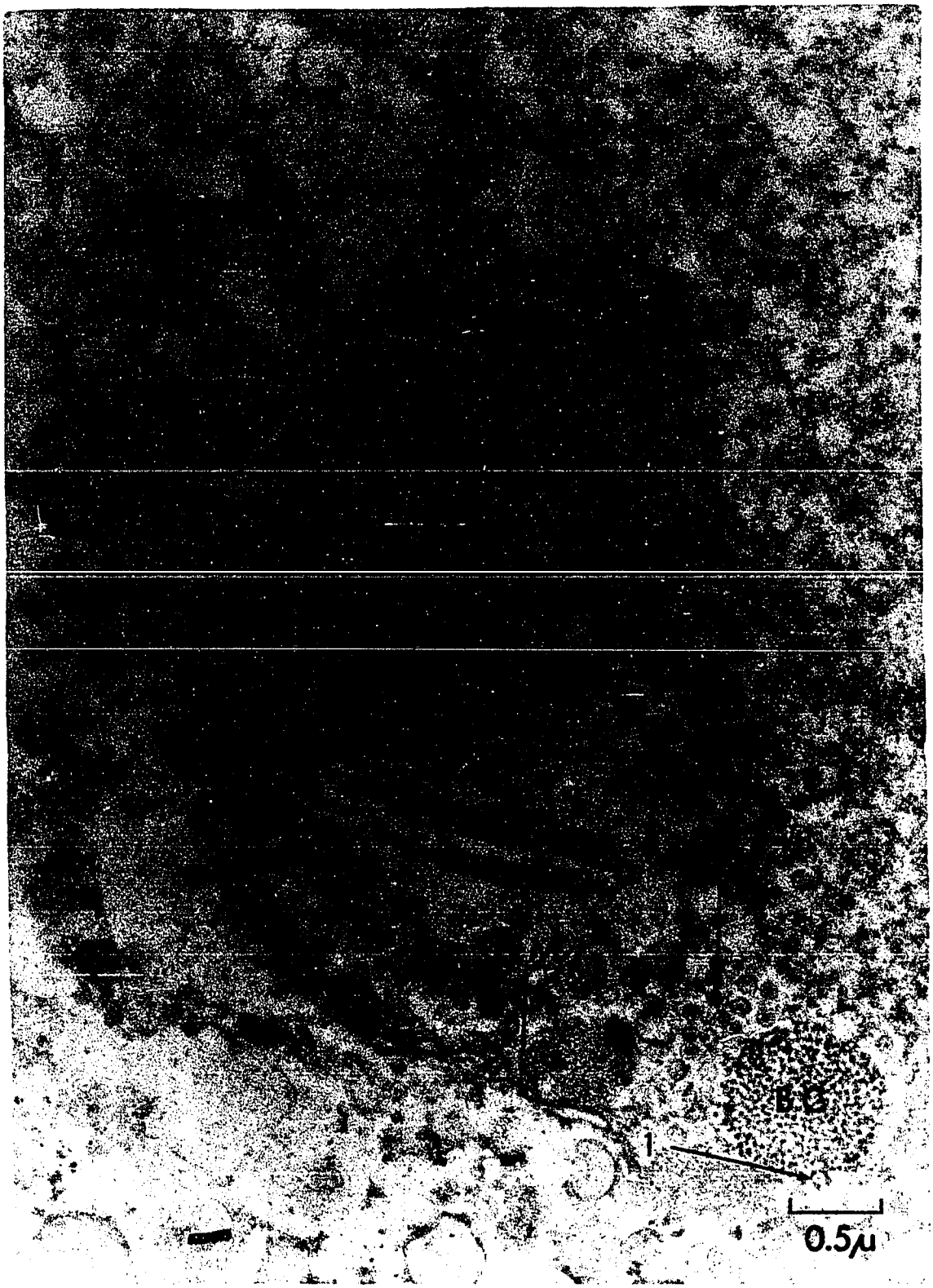
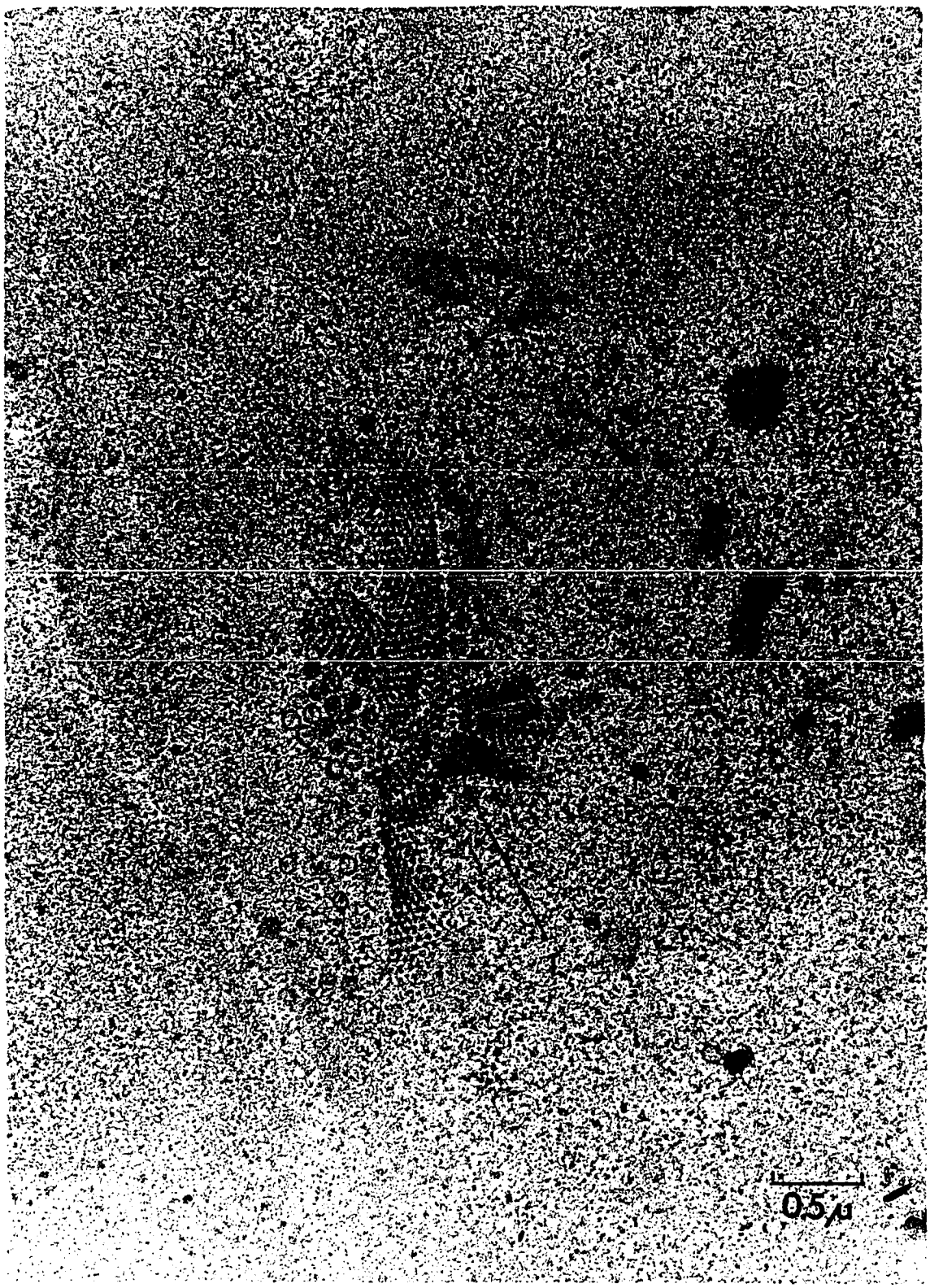

B. C.
1
0.5μ

Figure 6. FL Human Amnion Cells Infected with Herpes Simplex Virus. Forty Hours Post-Infection. Cells Grown in Presence of 4.31 mcg Human Growth Hormone per ml Medium.


0.5μ

nuclear membrane (NM). Within or closely associated with the marginated chromatin are a circular focus of B granules (BG), single membraned particles (SMP) with or without electron-dense cores (nucleoids), and two linearly arranged structures (LS) which are approximately 1.25 μ in length. LS structures appear to be composed of continuous fibrils alternating with spherical bodies. Peripheral to the B granules (1, Figure 5) a single membraned particle appears to be in process of forming. Figure 6 illustrates a perinuclear structure composed of single membraned particles, with and without electron-dense cores, closely associated with a crystalline array of electron-dense particles, tentatively termed "A bodies." These bodies (A, Figure 6) correspond in size and electron density (approximately 20 mμ in diameter) to the spherical bodies of the LS structures in Figure 5. A majority of particles appear to be parallel to plane of sectioning, but in the area (1, Figure 6) they appear perpendicular to plane of sectioning, suggesting that these particles may be in a plane of one particle in thickness.

Electron microscopic examination of all samples described in Table 16 revealed LS structures to be present only in cells pretreated with 10 mcg HGH, 29 hours post-infection (LS, Figure 7).

Effect of Human Growth Hormone on Uptake of Glucose by HSV Infected FL Human Amnion Cells

Inasmuch as HGH was shown to reduce uptake of glucose (Table 14) and infection with HSV results in increased uptake of glucose (Fisher and Fisher, 1961), an experiment was designed to determine what effect, if any, various concentrations of HGH had on uptake of glucose in HSV infected FL amnion cells. Results, expressed on a population basis in

Figure 7. FL Human Amnion Cell Infected with Herpes Simplex Virus. Twenty-nine Hours Post-Infection. Cell Grown in Presence of 10 mcg Human Growth Hormone per ml Medium.


0.5μ

Table 17, suggested that increasing concentrations of hormone result in decreasing uptake of glucose. A combination of HGH and anti-HGH antibody in the system resulted in glucose uptake similar to that of control.

Effects of p-Fluorophenylalanine on Replication of HSV in FL Human Amnion Cells

Media SMEM was changed on two logarithmically growing suspension cultures of FL amnion cells and populations were adjusted in each to 3.12×10^5 viable cells per ml media. Media of one flask was supplemented with 0.5 mM p-fluorophenylalanine (FPA), whereas the other flask, which did not contain FPA, served as control. Cells were immediately infected with 0.4 $TCID_{50}$ HSV per cell and samples for infectious virus titrations were removed at various times after infection. Results, as shown in Table 18, suggested that addition of 0.5 mM FPA to SMEM, which normally contains 0.2 mM phenylalanine, reduced yields of infectious virus by a factor of approximately 200-fold at 17 and 25 hours post-infection. Results also indicated that cells in presence of FPA were producing infectious virus at a constant rate between 18 and 41 hours after infection, whereas virus in control cultures reached maximum titers at about 25 hours post infection.

To determine whether or not time of addition influenced the yield of infectious virus, FPA (final concentration of 0.5 mM) was added two hours prior to infection and two hours after infection. In a third flask FPA was added at time of infection, but phenylalanine (PA) was adjusted from 0.2 mM to 1.0 mM (diluted from a sterile stock solution of 20 mM PA), which resulted in a molar ratio of 1 FPA:2PA. A comparison of the effects of adding FPA two hours prior to infection to two hours

TABLE 17

EFFECTS OF CONCENTRATION OF HUMAN GROWTH HORMONE ON THE DEPLETION OF D GLUCOSE BY FL CELLS INFECTED WITH HERPES SIMPLEX VIRUS

Concentration of Hormone	Hours Post-Infection		
	6.5	24.5	29.0
0.1	430[b]	150	120
1.0	420	230	200
10.0	450	220	250
10.0 + AHGH[c]	430	120	90
NRS[d]	480	116	90

[a]Expressed as mcg human growth hormone per ml medium. Cells grown in presence of hormone for 18 hours and subsequently infected with 0.4 $TCID_{50}$ HSV per cell.

[b]Mcg D glucose per ml supernatant medium. Content of medium at time of seeding was 1040 mcg. D glucose per ml medium.

[c]Rabbit anti-human growth hormone antibody incubated with human growth hormone 1 hour 37°C before adding to media.

[d]An equivalent volume of non-immune rabbit serum.

TABLE 18

EFFECTS OF p-FLUOROPHENYLALANINE ON REPLICATION OF HERPES SIMPLEX VIRUS IN FL AMNION CELLS GROWN IN SUSPENSION CULTURE

Hours Post-Infection[a]	Titers of Virus	
	Medium with FPA[b]	Control Medium without FPA
2.0	1.33[c]	1.77
17.5	3.00	5.23
25.5	3.50	5.87
41.5	3.33	2.31

[a] Cells infected with 0.4 $TCID_{50}$ HSV per cell at time 0.

[b] Final concentration of p-fluorophenylalanine adjusted to 0.5 mM at time of infection.

[c] Titer of virus expressed as positive $\log_{10}$ $TCID_{50}$ HSV per 0.1 ml medium.

post-infection (Table 19), indicated that addition prior to infection results in a 740-fold reduction of infectious virus production 46 hours post-infection. Increasing concentration of PA resulted in an increase of infectious virus formation. A comparison of infectious virus formation in presence of FPA added at time of infection (Table 18), added two hours after infection, or added two hours prior to infection (Table 19) suggested that, once infectious virus formation begins, the rate of synthesis remains fairly constant for a period up to 46 hours post-infection.

Six samples, numerically designated (Table 19) and removed at the same time as samples for determination of titers of infectious virus production, were examined with the electron microscope. Screening of samples 1, 3, and 5 revealed a pattern of similar development to that seen in virus infected control cultures.

In Figure 8 (a representative micrograph of sample 5) may be seen two foci of single membraned particles (SMP) and a number of double membraned particles (DMP) within the microvilli (MV) and cytoplasmic vacuoles (CV). In only one of eighty-two micrographs of samples 1, 3, and 5 were any linearly arranged structures encountered (LS, Figure 9 from sample 3). These structures appeared in different planes of orientation. Discontinuous bodies, alternating with continuous fibrils, appeared not as single spherical bodies as previously observed, but subdivided into two separate components.

Although linearly arranged structures were rarely encountered in samples 1, 3, or 5 (22 hours post-infection), they were found in many, but not all, cells from samples 2, 4, and 6. Gross observations

TABLE 19

EFFECT OF TIME OF ADDITION OF p-FLUOROPHENYLALANINE ON THE REPLICATION OF HERPES SIMPLEX VIRUS IN SUSPENSION CULTURES OF FL AMNION CELLS

Time of Addition of FPA[a]	Hours Post-Infection			
	22		46	
	TV[b]	EM[c]	TV	EM
Two hours prior to infection	2.00[b]	1[c]	1.78	2
Two hours post-infection	3.50	3	4.33	4
At time of infection[d]	4.50	5	4.83	6

[a] Final concentration of p-fluorophenylalanine 0.5 mM.

[b] Titer of virus expressed as positive $\log_{10}$ $TCID_{50}$ HSV per 0.1 ml medium. Cells infected at 0 time with 0.4 $TCID_{50}$ HSV per cell.

[c] Electron microscopy sample number.

[d] Phenylalanine concentration adjusted from 0.2 mM to 1.0 mM.

Figure 8. FL Human Amnion Cell Infected with Herpes Simplex Virus. Twenty-two Hours Post-Infection. 2 Phenylalanine:1 p-Fluorophenylalanine.

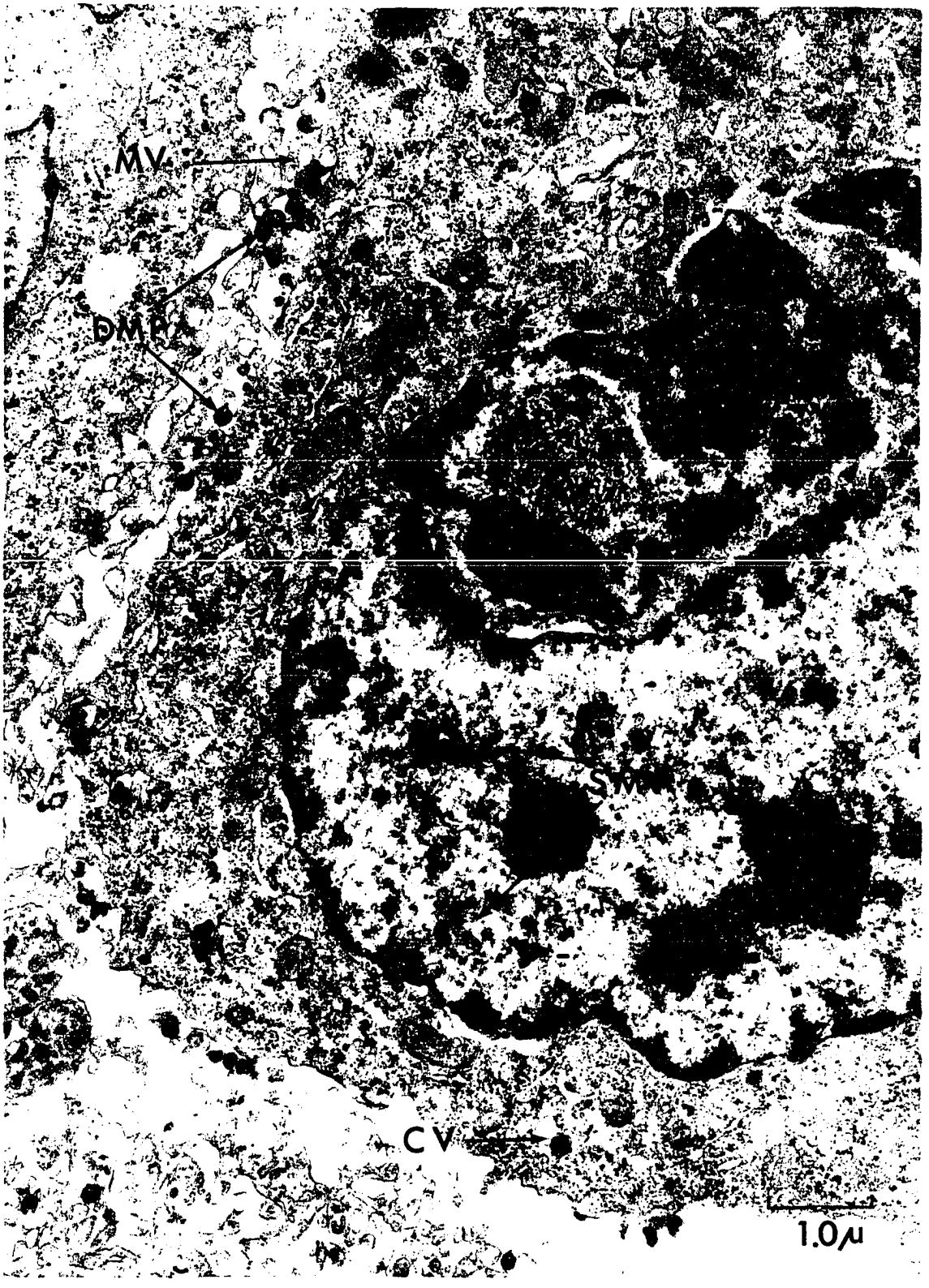
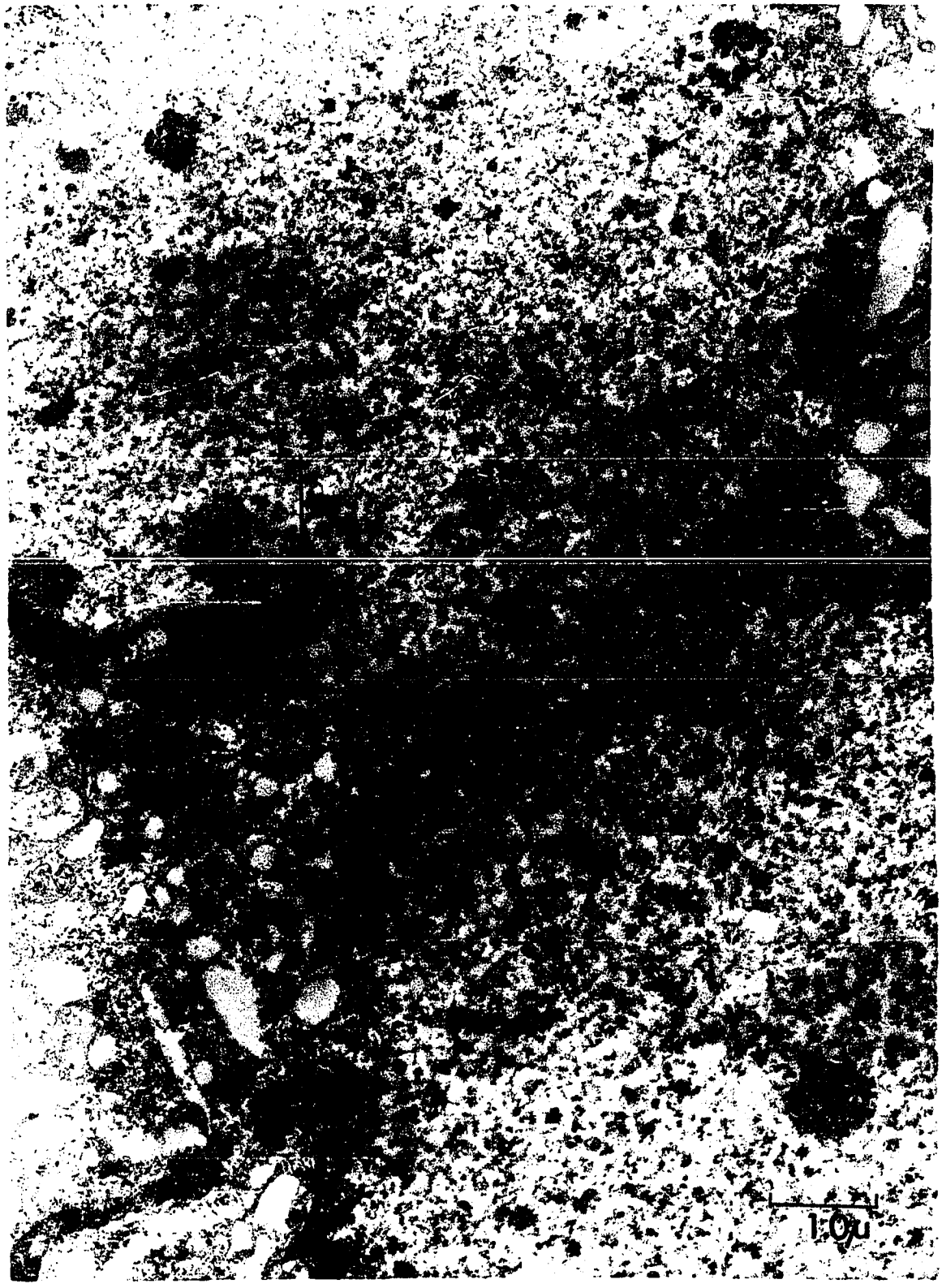

MV
DMPA
SM
CV
1.0μ

Figure 9. FL Human Amnion Cell Infected with Herpes Simplex Virus. Twenty-two Hours Post Infection. 2 Phenylalanine:1 p-Fluorophenylalanine.


1.0μ

indicated that the only apparent difference was that of increased quantity of LS structures and increased cytopathic effect on cells in sample 6, as compared to samples 2 or 4. Linear structures were observed in substantially every cell in sample 6, and therefore it was subjected to more extensive electron microscopic examination than were samples 2 or 4.

The following described micrographs, unless otherwise indicated, were taken from sample 6. Figure 10 shows a micrograph of low magnification (X4500) of a polykaryocyte. Numerous virus particles can be seen extracytoplasmically and within the nucleus and cytoplasm. Linearly arranged structures (LS) can be seen within nuclei and immediately adjacent to marginated chromatin. Extensive cytopathic effect is evidenced by marked vacuolization of cytoplasm and degeneration of mitochondria.

Several morphological forms which were only rarely observed are shown in Figure 11. Bundles of electron-dense, tightly interwoven fibers, which will be termed "A fibers" (AF), can be seen dispersed throughout the nuclear area. In several areas (1, Figure 11), these fibers appear to be fusing with marginated chromatin. Marginated chromatin (2, Figure 11) appears, not as a homogenous matrix as it does in other areas of the chromatin, but seems to have assumed a more lineate pattern. A linearly arranged structure (LS) and B granules (BG) are also present within the nucleus.

Figure 12 shows HSV in numerous stages of development. A number of single membraned particles (1, Figure 12) appear to be forming from a matrix of viral sub-units. Spherical bodies (2, Figure 12) correspond in size and electron density to A bodies seen in Figure 6. Immediately

Figure 11. FL Human Amnion Cell Infected with Herpes Simplex Virus. Forty-six Hours Post-Infection. 2 Phenylalanine:1 p-Fluorophenylalanine. Formation of "A Fibers".

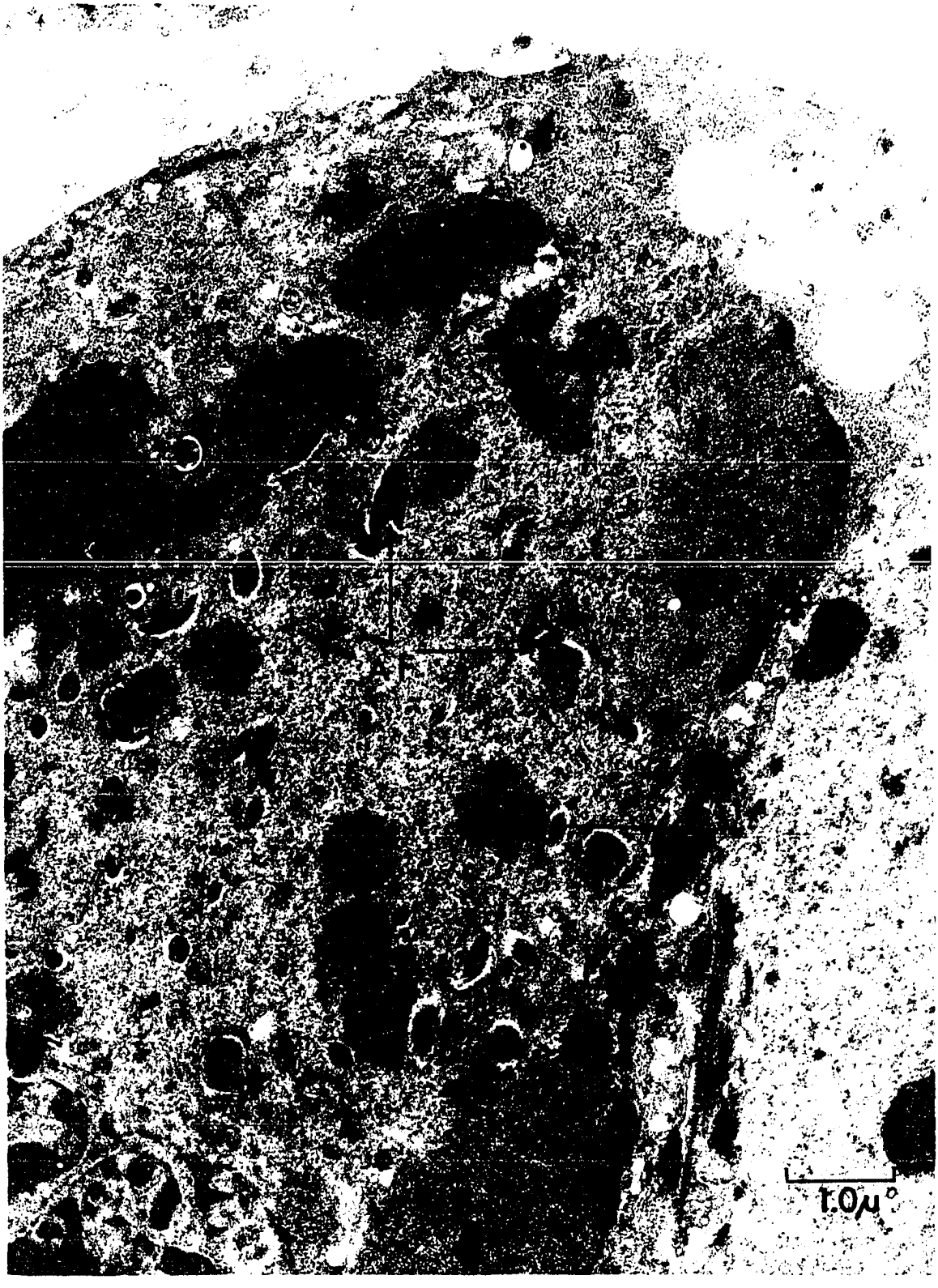
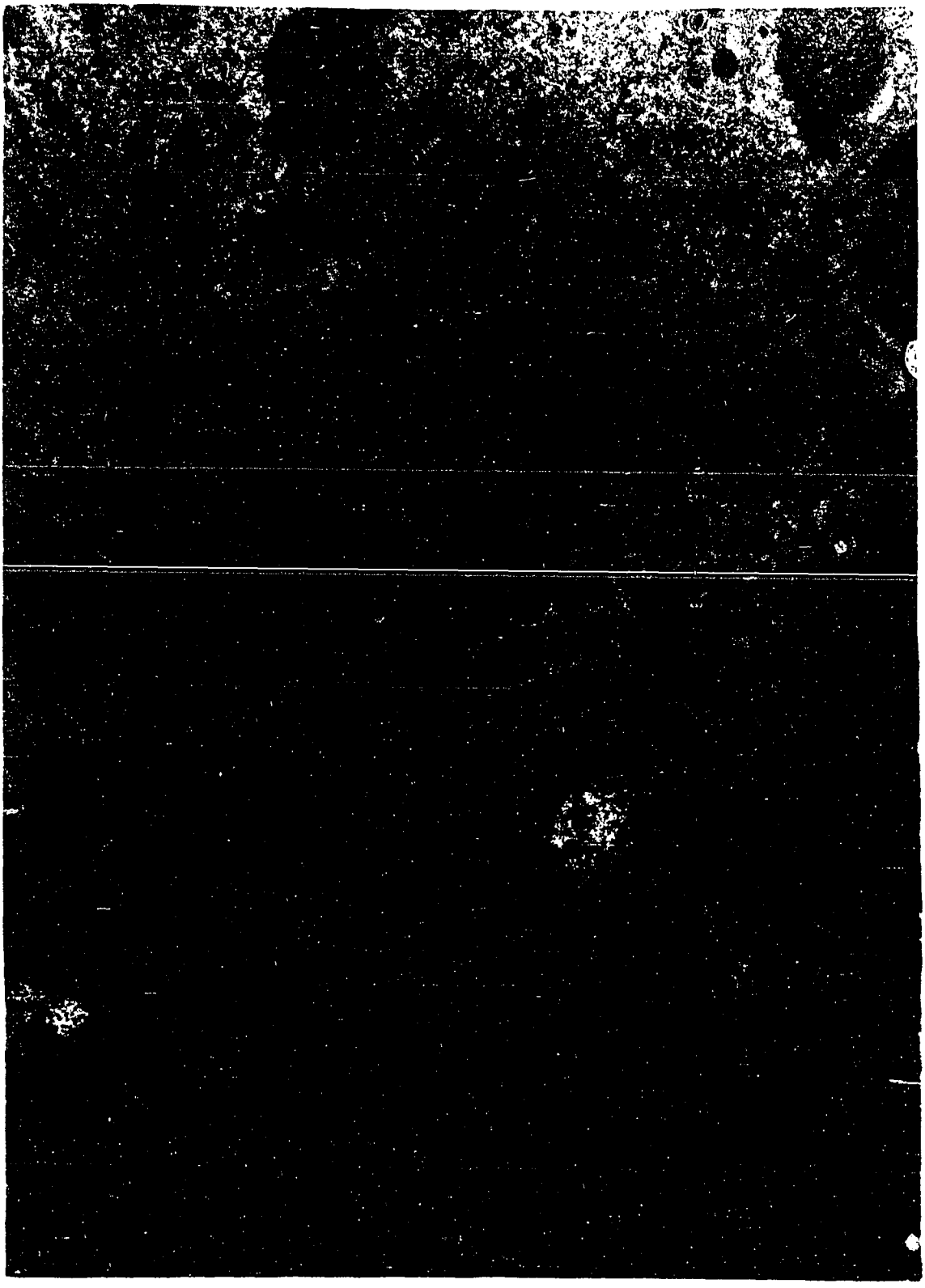

1.0μ

Figure 12. FL Human Amnion Cell Infected with Herpes Simplex Virus. Forty-six Hours Post-Infection. 2 Phenylalanine:1 p-Fluorophenylalanine. Viral Developmental Stages.



adjacent to lighter staining single membraned particles are particles which have acquired a more characteristic darker staining nucleoid. Transition from one type of staining to the other type generally occurs within a distance of one particle (approximately 90 mμ). A single membraned particle of low electron density appears hexagonal in form (3, Figure 12). Within perinuclear areas (4, Figure 12) single membraned particles appear to have finger-like projections around them.

Figure 13 depicts a focus of single membraned particles which appear to be in process of formation. Spherical bodies (1, Figure 13) correspond in size and electron density to those seen in Figure 12. The particle seen at 2 in Figure 13 appears to have been fixed at the time of sub-unit assembly. A majority of single membraned particles appear spherical in shape but in some instances they assume a more hexagonal shape (3, Figure 13). Many of the single membraned particles are devoid of nucleoids.

Details of the fine structure of a linearly arranged matrix of material are shown in Figures 14 and 15. Different planes of orientation (Figure 14) and similar planes of orientation (Figure 15), with respect to plane of sectioning, are evident. A linear progression along adjacent pairs of spherical A bodies (3, Figure 14) suggests a morphological transposition from paired units (1, Figure 14), to doubled paired units (2, Figure 14), and finally to coalesced spherical A bodies (3, Figure 14). When bodies exist in pairs (1, Figures 14 and 15), the term "diplosome" is tentatively suggested. Adjacent diplosomes always appear to be separated by a well-defined fibril. A comparison of spatial relationships between adjacent diplosomes suggest a longitudinal section

Figure 13. FL Human Amnion Cell Infected with Herpes Simplex Virus. Forty-six Hours Post-Infection. 2 Phenylalanine:1 p-Fluoro-phenylalanine. Assembly of Viral Sub-units.

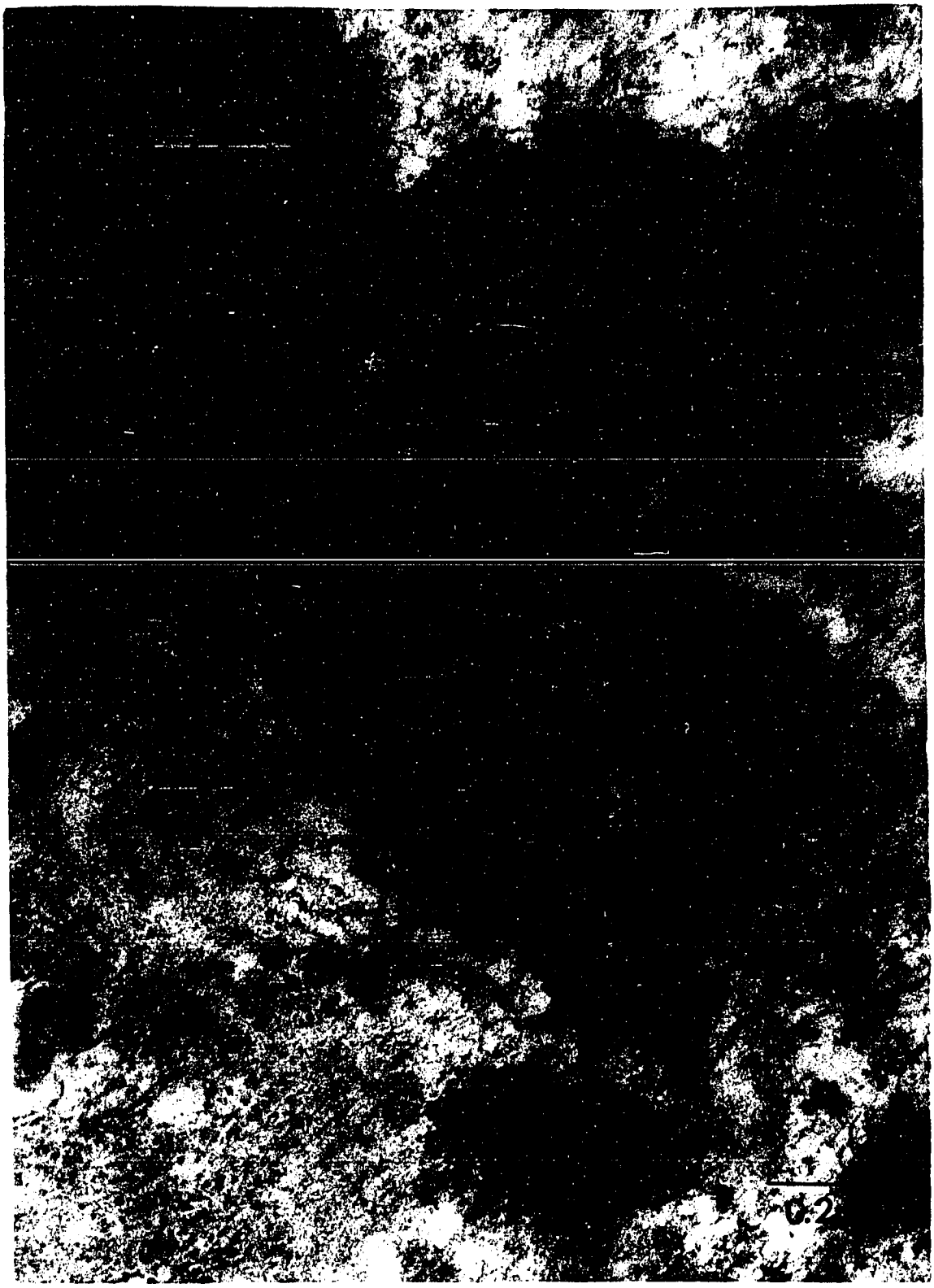
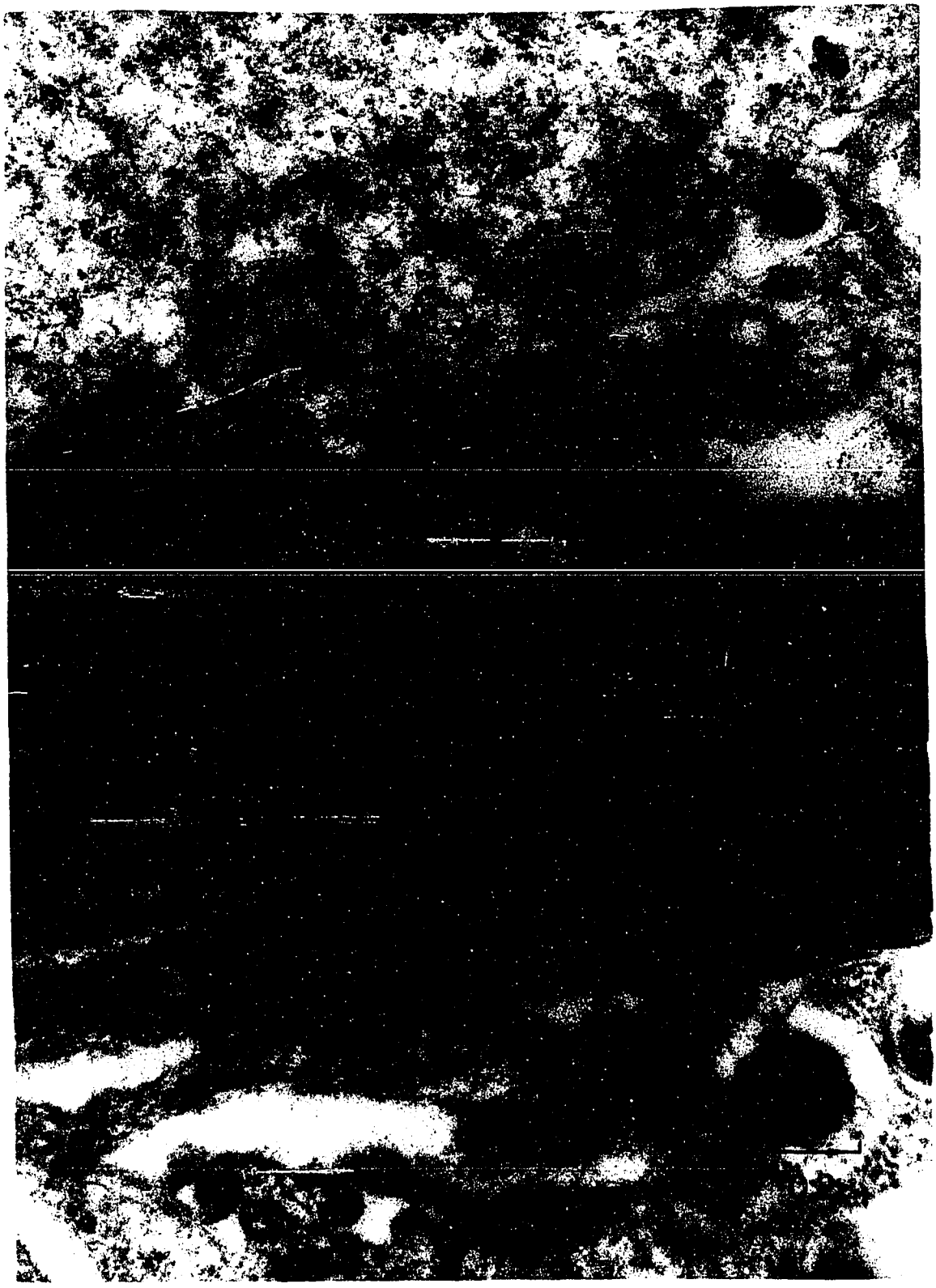

0.2

Figure 14. FL Human Amnion Cell Infected with Herpes Simplex Virus. Forty-six Hours Post-Infection. 2 Phenylalanine:1 p-Fluorophenylalanine. Linearly Arranged Matrix.

Figure 15. FL Human Amnion Cell Infected with Herpes Simplex Virus. Forty-six Hours Post-Infection. 2 Phenylalanine:1 p-Fluoro-phenylalanine. Linearly Arranged Matrix.



through a helical structure. In some areas (2, Figure 15) fibrils fuse and anastomose at acute angles with adjoining fibrils.

Examination of some cells revealed that A bodies may not disassociate from a linear fibril but remain associated to form long continuous strands of A bodies (1, Figure 16, and 3, Figure 15). Spatial arrangement between opposing A bodies likewise suggests a longitudinal section through a helical structure.

Effect of FPA on Glucose Utilization by HSV Infected FL Amnion Cells in Suspension Culture

It was considered of interest to determine whether FPA might effect energy metabolism of virus infected cells. For this purpose, FPA (final concentration of 0.5 mM) was added to one of two populations of FL amnion cells and five hours later both cultures were infected with 0.4 $TCID_{50}$ HSV per cell. At 11, 16 and 35 hours after infection, samples were removed from each flask and glucose present in supernatant media was determined. Results, as shown in Table 20, showed that the presence of FPA, within described experimental conditions, results in decreased utilization of glucose in HSV infected FL amnion cells.

Effect of FPA on Morphology of FL Amnion Cells

Inasmuch as some of the morphological forms seen in infected cells in presence of FPA might be attributed only to artifactual effects of this compound on cells, an experiment was designed to determine effects of FPA on non-infected FL amnion cells. Cells were incubated in presence of 0.5 mM FPA and samples were removed 21 and 40 hours after addition of FPA. No evidence of infection or formation of fibrils

TABLE 20

EFFECT OF FPA ON DEPLETION OF D GLUCOSE FL AMNION CELLS INFECTED WITH HERPES SIMPLEX VIRUS

Hours Post-Infection	Mcg D Glucose per ml Medium	
	Medium with FPA[a]	Medium without FPA
0	1040	1040
11	910	800
16	815	785
35	710	600

[a] p-fluorophenylalanine concentration 0.5 mM. Cells infected with 0.4 $TCID_{50}$ HSV per cell 5 hours after addition of FPA.

Figure 16. FL Human Amnion Cell Infected with Herpes Simplex Virus. Forty-six Hours Post-Infection. 2 Phenylalanine:1 p-Fluorophenylalanine. Continuous Strands of "A Bodies".



was seen in any of the examined sections. However, a representative micrograph from the 40 hour sample (Figure 17) suggests that incorporation of FPA does result in enlargement and bizarre morphology of mitochondria (M). In addition, cytoplasm of cells in presence of FPA appears more vacuolated than those not in the presence of this compound (Figure 2). Although not quantitated, gross observations suggested that FPA causes an increase in cell size.

Infection of FL Amnion Cells with HSV from Sample 6

To satisfy the question of whether HSV, after replication in a system containing FPA, would subsequently revert back to a pattern of development seen in infected control cultures, FL amnion cells were infected with 0.4 $TCID_{50}$HSV (Sample 6) per cell. The possibility of introducing the effect of FPA from sample 6 into the normal system was minimized by mixing an equal volume of 20 mM phenylalanine with sample 6 prior to infection. Samples for electron microscopy were removed 16 and 35 hours post-infection. No unusual structures were encountered in any of the examined sections. A representative micrograph (Figure 18) of the 35 hour specimen shows a pattern of development similar in morphology to that seen in control cultures (Figure 5).

Figure 17. FL Human Amnion Cell. Incubated in Presence of 0.5 mM p-Fluorophenylalanine for Forty Hours.

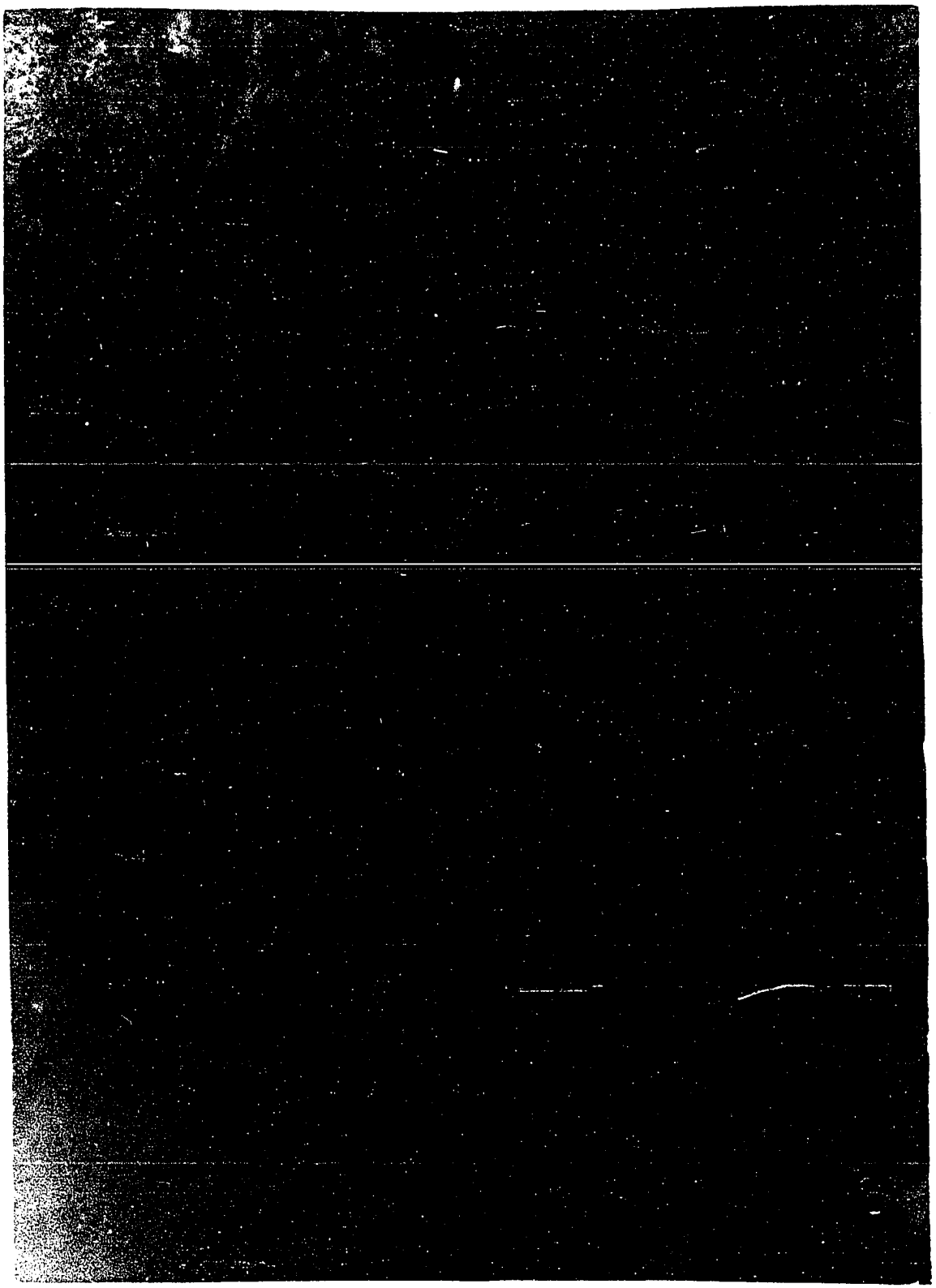
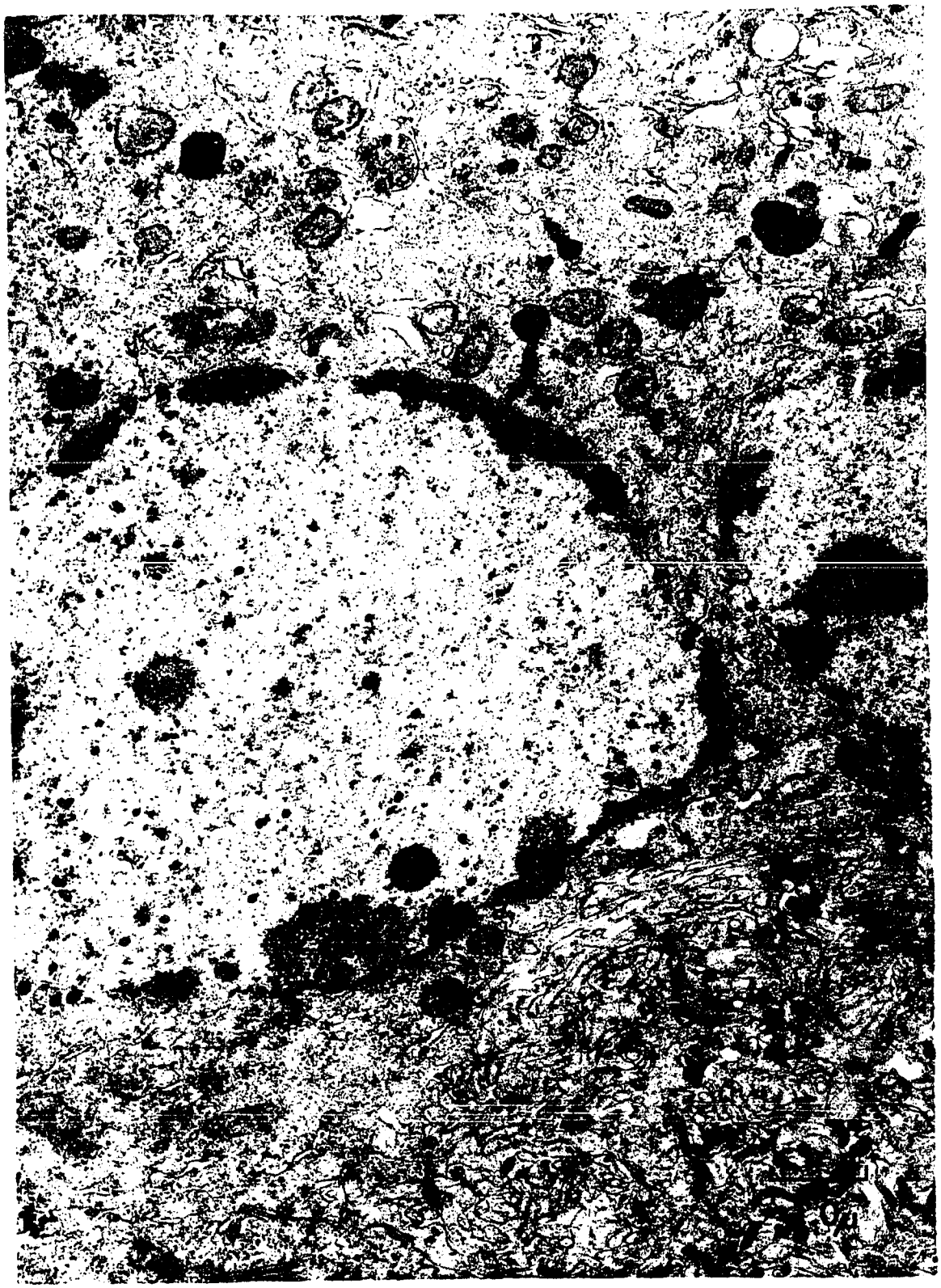


Figure 18. FL Human Amnion Cell Infected with Herpes Simplex Virus Previously Replicated in Presence of p-Fluorophenylalanine.



CHAPTER V

DISCUSSION

These studies were initially undertaken to determine the effects of hormones on replication of HSV in an in vitro system. It has long been appreciated that hormones act as potent regulators for the control of metabolism, however, modes of action are poorly understood. Synthesis of virus is dependent upon metabolism of the host cell, therefore alterations in the metabolic activities of cells should result in modifying the growth cycles of virus. This should be true especially if metabolic pathways common to both virus and cell are altered. Prior to the study of hormonal influence on virus-cell interaction, it was deemed necessary to direct studies toward determinations of the effects of several hormones on non-infected cells. Studies concerning effects of hormones at the cellular level and macromolecular synthesis of animal viruses are in the early stages of development. Therefore, parameters of study were limited to determining: (1) proliferation of cells as an index of total cellular metabolism; (2) content of cellular protein as an index of protein metabolism and; (3) depletion of glucose as an index of energy metabolism. Results of experiments in which multiplication of cells in the monolayer system several days post

seeding was examined as a function of concentration of hormone suggested that low concentrations of somatotropin were more stimulatory than higher concentrations (Table 1). In fact, higher concentrations of hormone appeared not to be stimulatory, but slightly inhibitory to cellular multiplication. This observed effect of inhibition could be attributed to toxicity of hormone in high concentrations. Monolayers were washed with saline and treated with trypsin prior to enumeration. Therefore, cell counts actually reflected only viable cells and inhibitory effects could be attributed to a temporal shift in the growth phase of cells. Cells grown in vitro normally exhibit a pattern of development characterized by a lag phase, a log phase of growth, a stationary phase, and a death phase. The possibility exists, therefore, that cells under influence of high concentration of hormone were in the death phase, and cells in the presence of low concentrations of hormone were in stationary or log phase of growth. In order to determine whether effects of hormone were a function not only of concentration, but also time, the effects of 2.5 mcg BGH per ml medium on multiplication of cells as a function of time were examined. Results of these experiments indicated that somatotropin decreases generation time of cells, resulting in an asynchronous phase of growth compared to control cultures. Therefore, sampling of cultures, with or without hormone, at the same chronological time results in sampling at different physiological times. Thus, the effect of somatotropin on cellular proliferation is one of marked stimulation, inhibition, or no effect, depending upon time of comparison with control cultures.

Serially propagated tissue culture cells require addition of protein to chemically defined media for growth. Inasmuch as growth of cells in vitro requires an exogenous supply of proteins, and generation time of cells is dependent to some extent upon concentration of serum, it was of interest to determine if effects of hormone were dependent not only on concentration of hormone, but also on concentration of serum which also contains hormone. Results of these studies showed that media supplemented with 2.5 mcg BGH and five or 10 per cent serum (Table 3) results in stimulation of cellular proliferation when compared to respective control cultures. The stimulation was more pronounced in media supplemented with five per cent sera than with ten per cent sera which suggests that somatotropin is more stimulatory on cells less metabolically active and therefore the degree of stimulation is correlated with the physiological state of the cell. It is interesting to note that rate of growth of cells in media supplemented with ten per cent calf serum or five per cent calf serum and 2.5 mcg BGH per ml medium are approximately the same. The possibility therefore exists that the ill-defined growth promoting activity of serum may be attributed, in part, to hormones present within the sera. A comparison of maximum populations of cells grown in media supplemented with five or ten per cent calf sera, with or without hormone, infers that although maximum populations attained are approximately the same, those under influence of hormone or increased concentration of serum have decreased time of generation.

Human growth hormone (HGH) was empirically shown to stimulate

proliferation of FL human amnion cells (Table 11). In view of this fact, it was anticipated that there might be a correlation between varying concentrations of hormone and response of cells. A comparison of FL cells grown in presence of 0.1, 1.0 or 10.0 mcg HGH per ml medium (Table 13) indicated that increased concentration of hormone results in increased proliferation of cells, but there did not appear to be a direct correlation between dosage and response. It is assumed that any improvement in the media may ipso facto result in increased growth of cells. Not only is cellular growth influenced by nutrients supplied by the media, but also by density of the population (Eagle and Piez, 1962). Therefore, it is assumed that stimulatory activity of HGH likely decreases with increasing density of the population.

Temporal studies with replicate tube cultures present the distinct disadvantage of sampling from different populations of cells; whereas such studies with suspension cultures present the advantage of sampling from the same population. A comparison between independent experiments showed that hormonal effect is certainly modified with respect to the initial population of cells. Therefore, minute differences in seeding of replicate tube cultures, and within the glassware itself,could modify response of cells to the hormone. These factors were as carefully controlled as possible in studies utilizing replicate tube cultures and results were the average of duplicate or triplicate experiments. However, it appears that the more feasible approach to temporal studies involves use of suspension cultures. Inasmuch as media, media supplements, and cells for each particular experiment were obtained from the same lot, then quantitative differences in response

of cells to hormone could be attributed only to effect of the hormone. However, comparisons between independent experiments resulted not only in comparing effects of hormone, but in addition, effects which could be attributed to initial phsyiological state of cells, initial population of cells, and variances in different lots of media and media supplements.

In one experiment (Table 13) the ability of rabbit anti-human growth hormone (AHGH) to neutralize the stimulatory effect of HGH on cellular proliferation was examined. Due to the possibility that antibody might not enter the cell, antibody and hormone were incubated together in vitro prior to addition to the system. Addition of this antigen-antibody complex (an equivalent of ten mcg HGH per ml medium) resulted in a population of cells intermediate in response to that of 0.1 or 1.0 mcg HGH per ml medium. Substitution of normal rabbit serum for HGH and AHGH resulted in a cellular response similar to ten mcg HGH and AHGH. It would thus appear that stimulatory activity of HGH is specifically inhibited by AHGH. Moreover, these results again suggested that an increase in concentration of serum (normal or immune) by five per cent modifies the stimulatory effect of somatotropin.

Effects of BGH on protein content of HeLa cells and of HGH on protein content of FL amnion cells (Tables 4 and 12) were examined. Results of these experiments indicated that somatotropin, in either system, induces increases in cell multiplication, but protein content of populations remains approximately the same as control cultures. Conversion of total protein per population to protein per cell

indicated that, in either system, the presence of somatotropin results in decreased content of protein per cell. These findings are in agreement with those reported by Moon et al. (1962). There appears to be striking differences in cell content of protein, RNA, and DNA during various phases of growth of cells (Salzman, 1959). HeLa cells are characertized by rapid synthesis of protein, DNA, RNA, and acid-soluble nucleotides during the lag phase. There seems to be a decrease of RNA and protein during logarithmic and stationary phases of growth. Addition of somatotropin tended to abbreviate the growth phase of cells, resulting in asynchronous phases of growth with respect to control cultures. This feature could, therefore, explain the findings of decreased content of cellular protein compared to control cultures.

Several investigations have shown that uptake of isotopically labelled amino acids into cellular protein is stimulated by somatotropin (Balis et al., 1958, and Prudden et al., 1956). If it is assumed that an effect of somatotropin on HeLa or FL cells may be similar to the findings of Balis and his group that on isolated microsomal ribonucleoprotein particles in stimulating incorporation of amino acids into protein, then a depletion of amino acid pools could result from cell-hormone interaction.

Lieberman and Owe (1959) reported that insulin enhanced proliferation of HeLa cells. An initial screening experiment with insulin in the present studies, the results of which appear in Table 5, indicated that insulin did not stimulate proliferation of cells, but, on the contrary, was slightly inhibitory. However, these studies were not pursued and may have reflected a temporal relationship in the

growth phase of cells similar to that noted with the somatotropin experiments. The reported stimulatory effect of insulin on cellular proliferation was noted in a system which contained partially purified serum protein. It is therefore possible that insulin activity is modified by unidentified growth promoting factors in the serum and activity of insulin might be additive, if examined in combination with such known growth promoting substances as somatotropin. Results of an experiment in which the effects on growth of HeLa cells of varying amounts of insulin and ten mcg BGH per ml medium are shown in Table 7. These data showed that a combination of insulin with BGH is more stimulatory on cellular proliferation than BGH alone. Lower concentrations of insulin proved to be more effective than higher concentrations. Protein content of cells grown in presence of five mcg BGH and 0.5 unit insulin per ml medium are shown in Table 8. These data again suggest, as with the use of BGH alone, that there is a marked decrease in amount of cellular protein per cell compared to control cultures at the same time.

Somatotropin and insulin in vivo do not appear to affect any particular target organ, but seem to produce a more generalized effect. Therefore, it was of interest to determine whether a hormone such as chorionic gonadotropin, which exhibits target organ effects in vivo, might stimulate growth of HeLa cells. Results of an experiment in which effects of five units chorionic gonadotropin (CGT) per ml medium on proliferation of HeLa cells are shown in Table 6. These results showed that CGT was not stimulatory and cells appeared to enter the death phase somewhat sooner than cells of control cultures. The effect of

BGH with varying concentrations of CGT on cellular proliferation was examined to determine whether CGT might not modify response to BGH. Results of these studies (Table 9) showed that, under these conditions, CGT was not stimulatory to cellular proliferation with any of the concentrations tested. To the contrary, it appeared that increasing concentrations of CGT resulted in decreased growth rates, decreased maximum population of cells, and earlier entry into the death phase.

Inasmuch as it had been shown that somatotropin stimulates proliferation of cells, it was expected that subsequent infection of metabolically stimulated cells with virus would result in an increased yield of infectious virus. Based upon the assumption that virus replication would proceed at a rate greater than would effects induced by hormones, various hormones were added to monolayers of cells 18 hours prior to infection with HSV. Two concentrations of HGH (five and ten mcg per ml medium) and CGT (five and ten units per ml medium) and one concentration of insulin (one unit per ml medium) were employed. Results of these studies (Table 10) suggested that temporal relationships existed between the control cultures and cultures with various hormones, with respect to the amount of infectious virus produced.

Results of an experiment to test the effect of chorionic gonadotropin and insulin on replication of HSV in monolayer cultures of HeLa cells are shown in Table 10. These studies were not pursued and are presented only as preliminary investigations. Production of infectious HSV in CGT treated cells appeared to follow similar temporal relationships with control cultures as was noted in HGH treated cells.

However, amount of infectious virus produced in insulin treated cells approached only a fraction of the amount produced in the control culture. Moreover, the morphology of HSV plaques was very distinctive in the insulin treated cultures. The morphology was characterized by the absence of macroplaques and polykaryocytes and the presence of numerous microplaques. Insulin utilized during these studies contained a preservative and zinc and it is therefore possible that reduction in virus synthesis could be attributed to toxicity of one or both of these substances. No cytotoxic effects were observed with insulin or insulin and somatotropin on non-infected cells (Tables 5 and 7). It is therefore possible that insulin, which is believed to alter permeability of cellular membranes (Krahl, 1961) may function in some unknown manner by which formation of giant cells or polykaryocytes is inhibited. These possibilities were not explored any further and are presented only as gross observations.

A comparison of infectious virus present in hormone treated and non-treated cultures four hours post infection, probably representing residual virus inoculum, showed that slightly less infectious virus was present in cultures under influence of five or ten mcg HGH and five units CGT. This effect possibly could be attributed to a slight increase in rate of adsorption and/or penetration of virus in the hormone treated cells. Since hormone treated cells are metabolically more active than control cells at time of infection, it would appear that the virus is eclipsed at a faster rate in hormone treated than non-treated control cells. Results suggested that cells pre-treated with HGH synthesize virus at a reduced rate. Peak infectious titers

attained with or without somatotropin were approximately equal, but maximum titers of infectious virus was attained approximately 24 hours sooner in the control cultures. It would, therefore, appear that total infectious virus produced in HGH treated cells is approximately the same as in control cultures, but rate of synthesis in HGH treated cells is reduced. A similar experiment in which FL cells were grown in suspension culture in presence of 4.31 mcg HGH per ml medium (Table 15) and subsequently infected with HSV, produced similar results. The HGH pretreated cells appeared to eclipse HSV at a greater rate than untreated cells and the rate of viral synthesis was reduced in HGH treated cells. Amount of virus present at any one time is a reflection not only of rate of synthesis but also of rate of thermal inactivation of the virus. Therefore, if the high rate of thermal inactivation of this virus is taken into consideration, as pointed out by Scott and McLeod (1959), it becomes apparent that a reduction in rate of synthesis but not amount of infectious virus detected, actually is a reflection of increased synthesis of virus in toto.

Since HGH was shown categorically to reduce rate of synthesis of virus. it was expected that the decrease in rate of snythesis of virus would be correlated with concentration of hormone. Moreover, if this effect is attributable to the specific activity of the hormone rather than to the non-specific activity resulting from the increased concentration of protein, then addition of anti-HGH antibody to the system should nullify the effects induced by the harmone. These possibilities were examined and results are shown in Table 16. It is apparent that an increase in concentration of HGH does result in a

decrease in rate of infectious virus synthesized, with the greatest reduction occurring in cells in presence of ten mcg HGH per ml medium. Addition of anti-HGH to the system appeared to nullify the effect of hormone and resulted in infectious virus production comparable to that produced in the control culture containing an equivalent amount of normal rabbit serum. Amount of virus present in the various cultures 6.5 hours post infection again suggested that virus is eclipsed by HGH treated cells at a faster rate than by non-treated cells or by cells in presence of HGH and anti-HGH.

Administration of somatotropin in vivo generally results in a diabetogenic state (Ikkos and Luft, 1959). It was of interest to determine whether the same effect could be observed at the cellular level. Influence of HGH on depletion of glucose by FL cells is shown in Table 14. Inasmuch as depletion of glucose is a function of population, results are reported as rate of depletion per cell. It is apparent that HGH treated cells utilize glucose at one-third to one-half the rate of non-treated cells. Depletion of glucose could be a reflection not only of increased population, but also of the physiological state of the cell. A comparison between populations of hormone-treated and non-treated cells indicates asynchronous growth cycles. If rate of glucose depletion were dependent only on the physiological state of the cell, then rate of depletion should vary with various growth phases of the cell. Rates of glucose depletion appeared to remain uniformly constant in hormone-treated and non-treated cells during comparable phases of growth, therefore reduced rate of glucose depletion found in this experiment could be attributed.

only to effect of hormone. Since there appears to be a stimulation of glucose utilization by tissue culture cells during infection with HSV (Fisher and Fisher, 1961), it was of interest to investigate whether or not HGH treated cells utilized less glucose than did non-treated cells when both were infected with HSV. Inasmuch as infection of cells with HSV results in necrosis of cells, it did not appear feasible to establish data on a per cell basis and therefore results of an experiment in which determinations of amount of glucose present in media of hormone-treated and non-treated cells at various times after infection are presented only as raw data in Table 17. Results of these experiments showed that treatment of FL cells with HGH prior to and during infection results in decreased utilization of glucose. Increased concentrations of HGH resulted in even greater decreased utilization of glucose. This effect was nullified when anti-HGH antibody was added to the system. It would therefore appear that pre-treatment of infected or non-infected FL cells with HGH results in decreased utilization of glucose. Since Fisher and Fisher (1961) reported that infection by HSV results in stimulation of the phosphogluconate oxidative pathway of carbohydrate metabolism, which is an important source of pentoses, it may be speculated that the observed reduction in rate of virus synthesis by HGH could be the result not only of a reduction in the availability of amino acids, but also of a reduction in the availability of pentose sugars necessary for nucleic acid synthesis.

Electron microscopic examination of HGH treated cells (Figure 1) and control cells without hormone (Figure 2) revealed normal cell structure characterized by well developed mitochondria and unorganized

endoplasmic reticulum similar to that reported by Fogh and Edwards (1959). Additional evidence of increased cellular proliferation mediated by HGH was afforded by the fact that mitotic figures were frequently encountered in hormone treated cells but not in control cells. It was observed, but not quantitated, that HGH treated cells frequently contained less cytoplasm than control cells without HGH.

Electron microscopic examination of FL cells infected with HSV forty hours post infection (Figures 3 and 4) revealed a pattern of development of virus similar to that reported by Morgan et al. (1958). Cytopathic effect of virus was characertized by margination of chromatin presence of single membraned particles (approximately 90 mμ in diameter) within the nucleus, and double membraned particles (approximately 120 mμ in diameter) within the cytoplasm. Foci of intranuclear granules within a polykaryocyte (Figure 4) were encountered in some, but not all, of the infected cells. The term "B granules" is suggested tentatively to denote these HSV specific granules, which are approximately 20 mμ in diameter, electron-dense, and closely associated with marginated chromatin and single membraned particles. Particles similar in morphology, size and location have been noted in HeLa cells infected with HSV (Morgan et al., 1959). An unusual micrograph of the classical Cowdry Type A intranuclear inclusion is shown in Figure 3, Although these inclusions are frequently observed by light microscopy in animal tissues infected with HSV, inclusion formation apparently has not been hitherto observed with the electron microscope in tissue culture cells. With one exception all specimens for electron microscopy were treated with trypsin prior to

fixation to remove cellular debris. Figure 3 is a representative micrograph from the specimen which was not treated with trypsin. Likewise, previous works by others have utilized trypsin in monolayer systems to remove cells from the glass substrate and therefore it is likely that cells with inclusions would not be encountered due to the digestive action of enzyme on grossly necrotic cells.

Comparisons between Figures 3 and 4 reveal considerable dissimilarities in cytopathic effect (CPE) even though specimens were infected with the same amount of virus (0.4 $TCID_{50}$ HSV per cell) and fixed at same time (40 hours post infection). Similar divergencies in CPE were observed within different cells from the same specimen. Low multiplicity of infection and the inability to infect all cells at the same time (Wildy et al., 1959) results in asynchronous development of virus within different cells. For many virus nutritional studies it is advantageous to infect as many cells as possible. However, it appears more practical to utilize low multiplicity of infections in studying the developmental stages of virus replication. Since low multiplicity of infection results in increased asynchrony of events within different cells, the probability of encountering different developmental stages of virus replication within the same specimen is greatly increased. Electron microscopic examination of FL cells infected with HSV in presence of 4.31 mcg HGH per ml medium revealed a pattern of development of the virus similar to that observed in non-hormone treated cells infected with virus, but in addition several unusual morphological forms were encountered. Inasmuch as these forms were seen only within HGH treated cells infected with HSV and never

within HSV infected control cultures (with HGH plus AHGH or without HGH) or non-infected control cultures (with or without HGH), it was believed that these forms were of viral etiology and the result of the influence of somatotropin on virus-cell interaction. The controls also ruled out the possibility that the observed phenomena were due to an adventitious infection by an unknown viral agent. Additional evidence that these forms are directly related to the synthesis of virus was suggested by the close association with them of focal accumulations of single membraned particles (Figure 6). Single membraned particles normally occur randomly dispersed throughout the nuclear matrix. It is assumed that focal accumulations of single membraned particles (SMP) are the result of rapid differentiation of virus at the site of synthesis. Therefore, morphological forms which are related to synthesis of this virus should be in close juxtaposition to focal accumulations of SMP. As shown in Figure 6, a number of single membraned particles are, indeed, closely associated with an array of spherical electron-dense particles.

Particles similar in size and morphology but separated by a continuous fibril are shown in Figure 5. A majority of the spherical particles appear orientated to plane of sectioning, however, within the area (1, Figure 6) the particles are not so oriented and appear tangential to plane of sectioning. At the apex (1, Figure 6) there appears to be some evidence of fibril formation. It is thus possible that whereas the structure indicated as LS in Figure 5 has been sectioned longitudinally; a cross section or end view of this structure is represented in Figure 6. If the primary unit of structure is

considered to be a continuous fibril with spherical granules on both sides, then the width of this primary unit as seen in longitudinal section should correspond to the distance between centers of spherical granules. Measurement of these distances indicate such a possibility.

Samples for electron microscopy and virus titrations were removed simultaneously from the materials indicated in Table 16. Of the 15 specimens examined only one with 10 mcg HGH at 27 hours post infection contained any linearly arranged structures (Figure 6). As was the case with infected cells with 4.31 mcg HGH, these structures were present in only a few of the examined cells. Inasmuch as these structures were present in the ten mcg HGH specimen but not in the ten mcg HGH plus anti-HGH antibody, induction of these forms could be attributed only to an influence of HGH on virus-cell interaction. It is also interesting to note that these structures were present at 29 hours but not at 24.5 hours post infection. Therefore, it would appear that not only must a sufficient amount of HGH be present (at least greater than 1.0 mcg per ml media) but also sufficient time must elapse after infection (at least 25 hours) before appearance of LS forms can be detected.

Location and electron density of these forms suggested that they contain nucleic acid compatible with virus nucleoids. If so, it would appear that processes of replication of HSV in HGH treated cells result in accumulations of virus nucleoids or precursors of virus nucleoids. Since it has been shown that the nucleoid of HSV is composed of DNA (Epstein, 1962), these morphological studies suggested that replication of viral DNA was proceeding at a faster rate than assembly of single membraned particles.

Darnell and Levintow (1960) have presented evidence that amino acids utilized in synthesis of poliovirus in HeLa cells comes from the intracellular free amino acid pool (de novo synthesis). It has been calculated that virus protein in this system comprises 0.1 to 1.0 per cent of total cellular protein and that free amino acids comprise approximately five per cent of the total amino acid content of the cell, and therefore a 90 per cent depletion of a single essential amino acid would still result in maximum synthesis of virus (Darnell et al., 1959).

It has been demonstrated that somatotropin stimulates incorporation of amino acids into cellular protein (Balis et al., 1958 and Prudden et al., 1956) and the possibility therefore exists that infection of cells with depleted amino acid pools could result in accumulations of virus nucleic acid due to a reduced rate of capsid formation. Since the present morphological studies indicated that electron-dense structures begin to accumulate approximately 25 hours after infection it may be assumed that sufficient amino acids were present for initial phases of the infectious process, but became depleted during later stages of infection. A depleted amino acid pool would account for a reduced rate of virus synthesis (Table 15) and the reduced amount of cellular protein present per cell (Table 12). As amino acids became available from the surrounding media, a full complement of infectious virus would be produced. The rate of virus synthesis would be governed by the rate at which amino acids became available from the media. If the foregoing interpretations are correct then similar results should be demonstrable by reducing synthesis of proteins by means other than with hormones.

It would appear that a minimum of two phases of virus synthesis requires synthesis of protein; namely, synthesis of enzymes and synthesis of viral proteins. The levels of three enzymes (DNA polymerase, RNA polymerase, and DNAase) increase shortly after infection (Russell et al., 1964 and Keir and Gold, 1963). Peak activity of complement fixing antigens is demonstrable later in the infectious cycle (Russell et al., 1964).

Therefore if synthesis of proteins is inhibited shortly after or at time of infection, synthesis of virus would likely be completely arrested. However, if synthesis of protein were allowed during early stages of infection and subsequently inhibited during later stages of infection, then results similar to those noted with somatotropin should be observed.

Studies utilizing p-fluorophenylalanine C^{14} (FPA) and phenylalanine (PA) have indicated that Bacillus cereus utilizes phenylalanine preferentially to p-fluorophenylalanine in the synthesis of proteins when levels of PA are high, but if the level of FPA is increased then FPA is incorporated into protein (Richmond, 1960). This amino acid analogue has been shown to inhibit synthesis of infectious virus (Zimmermann and Schafer, 1960, and Willcox and Ginsberg, 1961). Likewise, it has been demonstrated that inhibition of synthesis of infectious virus in presence of FPA is reversible upon removal of the analogue and addition of phenylalanine (Zimmermann and Schafer, 1960).

It would thus appear feasible that a correct combination of PA and FPA added to virus infected cells would preferentially utilize PA for synthesis of viral enzymes and subsequently result in a reduced

rate of synthesis during terminal phases of infection due to decreased levels of PA.

Based upon these assumptions, FPA (0.5 mM) was added to logarithmically growing FL cells in SMEM (0.2 mM PA) at time of infection with HSV. It was found, as indicated in Table 18, that this combination of PA and FPA did not inhibit synthesis of infectious virus but reduced yield of infectious virus 240-fold. Control cultures without FPA exhibited usual peaks in titer approximately 25 hours post infection, but FPA treated cultures appeared to maintain a constant rate of viral synthesis during the period of the experiments. Thus, as was the case with HGH treated cultures, a combination of PA and FPA resulted in a reduced rate of viral synthesis. Samples for electron microscopy studies were removed 25.5 and 41.5 hours post infection. Examination of the 25.5 hour specimen revealed a pattern of development similar to that noted in Figure 4, whereas examination of the 41.5 hour specimen revealed morphological forms similar to those present in Figures 5 and 6. The addition of 0.5 mM FPA to FL cells at time of infection with HSV resulted in an accumulation of linearly arranged structures in some, but not all, cells.

Since linearly arranged structures did not appear in all cells, the possibility existed that addition of 0.5 mM FPA at time of infection was not the optimal condition for induction of these forms. To test this possibility, time of addition of FPA with respect to time of infection, and the molar ratio of FPA:PA were altered. Results are shown in Table 19. It would appear from these studies that, regardless of whether FPA is added at time of infection, two hours prior to infection,

or two hours after infection, rate of synthesis of infectious virus remains essentially constant. Only total amounts of infectious virus produced are altered. Changing molar ratio of FPA:PA from 0.5:0.2 to 0.5:1.0 resulted in increasing rate of virus synthesis. It would therefore appear that rate of synthesis of infectious virus is dependent upon molar ratio of FPA:PA as well as time of addition of FPA, with respect to time of infection.

As previously mentioned, samples for electron microscopy (designated numerically) and for titration of infectious virus were removed at the same time (Table 19). Electron microscopic examination of samples removed 22 hours post infection revealed, in general, a normal pattern of virus development and maturation (Figure 8). Marked margination of chromatin, several foci of single membraned particles within the nucleus, and double membraned particles within the cytoplasm and cytoplasmic microvilli were evident. In only one cell (Figure 9) were any linearly arranged structures observed (22 hours post infection, Sample 3). Examination of samples 2, 4, and 6 revealed an abundance of linearly arranged structures. No qualitative differences in these structures were noted in samples 2, 4, or 6, but quantitative differences were noted. Linearly arranged structures were present in almost every cell from sample 6. Fewer of these structures were observed in samples 2 and 4. It would thus appear that a molar ratio of 1:2 (FPA:PA) is more nearly optimal for induction of these forms than is the ratio of 2.5:1 (FPA:PA).

Linearly arranged structures were easily detected in sample 6 and therefore it was subjected to the most intensive investigation.

As shown in Figure 10, linearly arranged structures were always found closely associated with marginated chromatin. This constant finding suggests, but does not prove, that these structures are derived from the marginated matrix.

On several occasions bundles of electron-dense and tightly interwoven fibers (AF, Figure 11) were observed within the nucleus. These fibers are tentatively designated as "A fibers." Since only a few single membraned particles were present within this particular nucleus, it is assumed that this area represented early stages of virus development. Acridine orange staining of HeLa cells infected with HSV has indicated that cells undergo a complete breakdown of particulate nuclear DNA, which is deposited as diffuse DNA in the perinuclear region of the cell (Ross and Orlans, 1958). A possibility, therefore, exists that this micrograph represents the same phenomenon, except at a different level of resolution. The supposition that host nucleic acid is degraded as a result of infection with HSV is further justified by an observed increase in DNAase activity (Keir and Gold, 1963), an increase in deoxyribonucleotides derived from host nucleic acid (Wildy et al., 1961), and a deposition of pre-labelled host nucleic acid in the marginated chromatin area (Munk and Sauer, 1964).

In several areas represented at "1" in Figure 11, "A fibers" appear to be fusing with marginated chromatin. If these "A fibers" represent partially degraded host DNA, it is difficult to explain why cellular DNA was not encountered in this morphological form in non-infected cell controls. Inasmuch as cellular DNA is combined with a histone protein, the possibility exists that substrate sites are not

available to the DNAase during interphase. However, during mitosis of the cell these sites could become available to the activity of the DNAase. Inasmuch as only 10 to 35 per cent of cells initially infected with HSV release virus (Wildy et al., 1959), it is possible that initial stages of the infectious process are limited by mitotic phases of the cell. Likewise, this would explain the failure of cells infected with HSV to divide (Wildy et al., 1961).

Figure 12 represents a composite of developmental stages of virus seen only in fragments in numerous other micrographs. Studies by Epstein and Holt (1963) and Wildy and Watson (1962) have suggested that the second, or outer, membrane of HSV is not virus-specific antigen, but is derived from protein of the host cell. No visual evidence has been presented to indicate mode of release of virus from the nucleus (Morgan et al., 1959).

In the perinuclear areas (4, Figure 12) finger-like projections can be seen around single membraned particles, suggesting formation of double membraned particles. It would thus appear that single membraned particles bud through the nuclear membranes and in so doing the nuclear membranes of the host cell form the second membrane of the virus particle.

In close association with spherical bodies (2, Figure 12) single membraned particles appear to be forming. The abrupt transition from spherical bodies (measuring approximately 20 mμ in diameter) to single membraned particles (measuring approximately 90 mμ in diameter) suggests rapid assembly of single membraned particles from a matrix of viral subunits. It would appear highly unlikely that juxtaposition of

spherical bodies and single membraned particles represent merely a casual relationship. If this were the case, then single membraned particles would not always be aligned in the same plane, but would be more randomly dispersed.

Greater detail in the relationship of spherical bodies and single membraned particles is depicted in Figure 13. A careful examination of these structures (1, Figure 13) suggests that single membraned particles are formed from adjacent pairs of linearly arranged bodies. Numerous particles are devoid of nucleoids, perhaps representing either faulty assembly of sub-units or sectioning which did not traverse the nucleoid.

Studies by Wilcox and Ginsberg (1963) and Flanagan and Ginsberg (1962) of adenovirus have shown that addition of FPA during late stages of the infectious process does not result in inhibition of synthesis of macromolecules but does result in faulty assembly of virus. FPA is incorporated into protein (Richmond, 1960). It is possible, therefore, that the capsid protein has incorporated FPA and results in mute physiochemical changes in the viral protein, thus leading to faulty assembly of single membraned particles. Since particles appear with and without nucleoids, it may be assumed that sufficient nucleic acid and "normal" viral protein were present concomitantly within the matrix for assembly of at least some complete (with nucleoids) particles. It would appear that faulty assembly is an exaggeration of a process in cells without FPA, since Wildy and Watson (1962) have shown that approximately 20 to 30 per cent of single membraned particles are assembled without nucleoids.

Linearly arranged structures were observed most frequently in different planes of orientation (Figure 14), but on occasion these structures were observed parallel o plane of sectioning (Figure 15), which would tend to support the view that replication of viral sub-units can occur at different levels within the nucleus. These structures invariably were observed in close association with marginated chromatin which supports, but does not prove, the supposition that linearly arranged structures are derived from precursor material in the marginated chromatin.

Upon higher magnification and within certain areas of a matrix of linearly arranged fibrils an interesting formation of paired viral sub-units appear regularly in the micrographs, as represented at "1," Figures 14 and 15. When sub-units exist in this form the term "diplosome" is tentatively suggested. Diplosomes are always separated longitudinally by well-defined fibrils. A linear progression along adjacent pairs of diplosomes indicates a morphological transposition from diplosomes into "tetrads," which then coalesce into large "A" bodies" (1 and 2, Figure 14). If diplosomes are considered replicating entities of this virus, it would appear that these viral sub-units (1, Figure 13) are the result of a self-duplicating unit. This unit appears to duplicate itself at least once and possibly twice.

Spatial configurations of opposing diplosomes (1, Figure 15) and spherical A bodies (Figure 16) separated by fibrils suggest the possibility of sectioning through a helical structure.

Accurate three-dimensional reconstruction of these morphological units from two-dimensional micrographs can be attained only by

sequential examination of serial sections. The existence of helical structures must remain purely speculative at this time, however, it is expected that this problem may be resolved in later studies.

Only indirect evidence suggests that fibrils contain DNA. Delineation of biochemical composition of these structures can be ascertained only by selective enzymatic digests of serial sections of the cells, particularly with proteinases, RNAase, and DNAase. A comparison by electron microscopy with each other and with undigested control sections should provide the means for inferential identification of structures. Electron density and location of these structures certainly suggested that they contain nucleic acid. Moreover, the content of DNA of this virus (Russell, 1962) and assembly of complete single membraned particles from these highly organized morphological structures would seem to indicate that these structures are composed of viral DNA and viral protein.

Disassociation of biochemical events by FPA and 5-fluoro-deoxyuridine in tissue culture cells infected with vaccinia (Salzman, 1963) has suggested that viral protein and viral DNA are synthesized at approximately the same time. It would appear likely that not only must these two components of infectious virus be synthesized simultaneously, but also within the same cellular location.

Fluorescent antibody studies have indicated that viral antigen of HSV is detected first within the nucleus (Lebrun, 1956). It would appear likely that structures seen in the present studies would allow synchronous synthesis of viral nucleic acid and viral protein within the same matrix. Early degradation of the nucleoli and

synthesis of RNA polymerase (Russell et al., 1964) indicated an intermediate role of RNA in snythesis of this virus.

If viral DNA and viral protein are synthesized within a linearly arranged matrix, it would seem probable that RNA would be required for transcribing genetic information into viral protein. Since A bodies appear to be the product of a duplicating unit, it is possible that the continuous fibrils separating these bodies may be composed of ribonucleoprotein.

Linearly arranged structures were observed in HGH and FPA treated cells infected with HSV. These structures were not observed in FPA treated cells (Figure 16), HGH treated cells, non-treated cells infected with HSV, or HGH + AHGH treated cells infected with HSV.

If linearly arranged structures were observed only in FPA treated cells infected with HSV, then it would seem possible that these structures may represent abnormal synthesis of viral macromolecules due to the incorporation of FPA into protein. However, since a normal biological material (HGH) appears to induce identical morphological forms, it may be assumed that these structures are a product of normal but reduced biosynthetic pathways of viral replication. If linearly arranged structures represent the product of normal but reduced physiological processes, failure to demonstrate these forms in any of the infected control cultures may be attributable to the following: (1) replication of viral DNA and encapsulation of HSV proceed at a rate (Roizman et al., 1963), which would likely prevent focal accumulations of viral macromolecules; (a) if rate of synthesis of HSV is governed by rate of synthesis of viral protein similar to that noted with vaccinia

(Salzman et al., 1963), it would appear highly unlikely that availability of amino acids from the metabolic pools would be depleted to a point to allow anything but maximum synthesis of virus (Darnell et al., 1959); and (3) based upon size of sample examined with the electron microscope (Morgan et al., 1959) the probability of encountering an isolated phenomenon is infinitesimal.

It may be speculated that pretreatment of cells with HGH results in partial depletion of amino acid pools due to increased incorporation of amino acids into cellular protein (Balis et al., 1958, and Prudden et al., 1956). Subsequent infection of these pretreated cells results in maximum synthesis of viral induced enzymes (an increase in levels of existing enzymes or de novo synthesis of novel enzymes) from existing amino acid pools, but as these pools become depleted, insufficient protein (either capsid protein or enzymes for assembly of macromolecules) is available for the assembly of complete viral particles. In time, as these amino acids become available from the surrounding media, normal maturation and development will occur.

Similarly, these cells may preferentially utilize phenylalanine (Richmond, 1960) during initial stages of the infectious process for maximum synthesis of viral induced enzymes. However, during later stages of synthesis of virus, substitutive incorporation of FPA for PA into viral protein and/or novel enzymes results in marked accumulation of unassembled viral macromolecules.

Negative staining technique has indicated HSV to be an icosahedral virus (Wildy et al., 1960). Studies of thin sectioned material have indicated that single membraned particles of HSV do not

exhibit the hexagonal appearance of an icosahedral virus, but appear spherical in shape (Morgan et al., 1959). However, examination of Figures 12 and 13 revealed a number of hexagonal shaped particles. The possibility existed that hexagonal configuration of the particles could be attributed to a compression from adjoining particles (Figure 13), but examination of other micrographs (i. e., 3, Figure 12) indicated well isolated particles with a hexagonal configuration. In general, particles which appear hexagonal in shape are characteristically light in electron density, which suggest that they are the more immature particles.

Due to the decreased utilization of glucose by FL amnion cells infected with HSV in the presence of HGH, it would seem possible that reduced rate of synthesis of virus could be attributed to a decrease in the phosphogluconate oxidative pathway if synthesis of appreciable amounts of de novo deoxyribonucleotides were utilized for synthesis of this virus. It would seem unlikely that the decreased utilization of glucose by FPA treated FL cells infected with HSV could in toto account for the marked reduction in synthesis of infectious virus.

Visual evidence presented in these studies and by Wildy et al. (1959) and Ross and Orlans (1958) suggest that infection of cells with HSV results in degradation of host DNA. Also, the high guanine and cytosine (68 per cent) content of DNA of HSV (Russell and Crawford, 1963) associated with the degradation of thymidine to CO_2 (Chang and Liepins, 1962) following infection of cells with HSV would suggest that large quantities of deoxyribonucleotides for synthesis of viral DNA are derived from the enzymatic degradation of host DNA.

Studies by Smith (1963) have indicated that aggregates of free HSV particles are connected by strands of deoxyribonucleic acid in such a way to suggest structural continuity between their cores which would imply that HSV particles are assembled from stands of DNA. Replicating fibrils seen in these studies are approximately 16 to 20 mμ in diameter. Based upon a diameter of 20 A^{o} of the DNA helix (Watson and Crick, 1953), it would appear that fibrils are composed of a maximum of eight to ten DNA molecules.

Electron microscopic examination of HSV replicated in presence of FPA and reintroduced back into a system without FPA (Figure 18) indicated that the virus reverts back to a normal pattern of development without any evidence of unusual morphological forms. It would thus appear that no genetic changes are induced by replication of virus in presence of FPA.

A number of perplexing questions concerning these studies remain unanswered. Among these, preliminary studies indicated that insulin reduced extracellular D-glucose with a concomitant decrease in replication of infective virus. These effects may be attributable to the preservative and zinc in the insulin. However, use of a crystalline type of insulin should distinguish between specific activity of hormone and non-specific activity of contaminating material. If reduction in infective virus is attributed to insulin alone, an approach similar to that used in somatotropin studies could be utilized.

No evidence has been presented to indicate if the "preliminary forms" are infective. Inasmuch as there is considerable difference in size of preliminary forms, single membraned particles, and double

membraned particles, then separation of these different forms by density gradient centrifugation and subsequent determination of infectivity of each component should be feasible.

Isotopically labelled FPA associated with autoradiography techniques should provide evidence to indicate if incorporation of FPA into capsid protein results in assembly of coreless particles. Anti-herpesvirus antisera and anti-human growth hormone antisera conjugated with ferritin could be used advantageously in locating (1) the sites of hormonal influence and (2) the sites of virus protein synthesis.

CHAPTER IV

SUMMARY

Somatotropin was found to stimulate the proliferation of FL human amnion and HeLa cells. The degree of stimulation of cells by the hormone was correlated with the concentration of hormone, time of incubation, and concentration of serum in the media. Cells under influence of somatotropin utilized less glucose and a concomitant decrease in content of cellular protein per cell was noted.

Subsequent infection of these metabolically stimulated cells with herpes simplex virus (HSV) resulted in a decreased rate in production of infectious virus. As concentrations of hormone were increased, rates of synthesis of infectious virus decreased. Addition of somatotropin plus anti-somatotropin antibody nullified the effects induced by the hormone.

Pathological cellular lesions of control cells infected with HSV consisted of marginated chromatin, scattered virus particles within the nucleus and cytoplasm, small foci of medium sized granules, and intranuclear Cowdry Type A inclusions. Examination of infected cells in presence of somatotropin revealed, in addition to the forms observed infected control cultures, crystalline arrays of spherical bodies (approximately 20 mu in diameter) sometimes separated by continuous fibrils.

Single membraned particles appeared to be forming from the crystalline arrays of sub-units. It was conjectured that a depleted metabolic pool of amino acids induced by the hormone resulted in a reduced rate of virus synthesis and the formation of incomplete virus particles lacking capsids.

A combination of phenylalanine (PA) and p-fluorophenylalanine (FPA) was added to virus infected cells to test this hypothesis. Incorporation of FPA with PA into the same system resulted in a reduction in rate of synthesis of virus. Molar ratio of PA:FPA and time of addition of FPA with respect to time of infection with virus governed the rate of virus synthesized.

In addition, FPA in combination with PA added to tissue culture cells infected with HSV resulted in marked accumulations of primitive and intermediate forms of virus.

Bundles of electron-dense and tightly interwoven fibers, tentatively designed as "A fibers" were observed fusing into the marginated chromatin. It was speculated that "A fibers" were the result of enzymatic degradation of cellular DNA. Many micrographs contained linearly arranged matrices consisting of paired sub-units, tentatively designated as "diplosomes," which were always separated longitudinally by well-defined fibrils. Diplosomes appeared to give rise to spherical bodies, measuring approximately 20 mμ in diameter, and tentatively designated as "A bodies" from which single membraned particles appeared to be assembled. Single membraned particles were observed in the perinuclear areas of the cell with finger-like projections surrounding them, suggesting that the nuclear membranes of the host cell may form the second membrane of the virus particle.

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